

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : G01N 33/576, C07K 15/00		A1	(11) International Publication Number: WO 91/15771
			(43) International Publication Date: 17 October 1991 (17.10.91)
(21) International Application Number: PCT/US91/02225		(81) Designated States: AU, BB, BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CM (OAPI patent), FI, GA (OAPI patent), GB, HU, JP, KP, KR, LK, MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NO, PL, RO, SD, SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).	
(22) International Filing Date: 29 March 1991 (29.03.91)			
(30) Priority data: 504,352 4 April 1990 (04.04.90) US			
(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: HOUGHTON, Michael ; 53 Rosemead Court, Danville, CA 94526 (US). CHOO, Qui-Lim ; 5700 Fern Street, El Cerrito, CA 94530 (US). KUO, George ; 1370 Sixth Avenue, San Francisco, CA 94112 (US).			
(74) Agents: CIOTTI, Thomas, E. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025-3471 (US).			
(54) Title: COMBINATIONS OF HEPATITIS C VIRUS (HCV) ANTIGENS FOR USE IN IMMUNOASSAYS FOR ANTI-HCV ANTIBODIES			
<div style="text-align: center;"><p>NH₂ — [C] — [E] — [NS1] — [NS2] — [NS3] — [NS4] — [NS5] — COOH</p><p>1 120 400 1050 1640 2000 3011</p></div>			
(57) Abstract			
<p>Combinations of HCV antigens that have a broader range of immunological reactivity than any single HCV antigen. The combinations consist of an antigen from the C domain of the HCV polypeptide, and at least one additional HCV antigen from either the NS3 domain, the NS4 domain, the S domain, or the NS5 domain, and are in the form of a fusion protein, a simple physical mixture, or the individual antigens commonly bound to a solid matrix.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

-1-

5

COMBINATIONS OF HEPATITIS C VIRUS (HCV) ANTIGENS
FOR USE IN IMMUNOASSAYS FOR ANTI-HCV ANTIBODIES

10

Description

Technical Field

15 The present invention is in the field of immunoassays for HCV (previously called Non-A, Non-B hepatitis virus). More particularly, it concerns combinations of HCV antigens that permit broad range immunoassays for anti-HCV antibodies.

20 Background

The disease known previously as Non-A, Non-B hepatitis (NANBH) was considered to be a transmissible disease or family of diseases that were believed to be viral-induced, and that were distinguishable from other
25 forms of viral-associated liver diseases, including that caused by the known hepatitis viruses, i.e., hepatitis A virus (HAV), hepatitis B virus (HBV), and delta hepatitis virus (HDV), as well as the hepatitis induced by cytomegalovirus (CMV) or Epstein-Barr virus (EBV). NANBH
30 was first identified in transfused individuals. Transmission from man to chimpanzee and serial passage in chimpanzees provided evidence that NANBH was due to a transmissible infectious agent or agents. Epidemiologic evidence suggested that there may be three types of NANBH:
35 a water-borne epidemic type; a blood-borne or parenterally transmitted type; and a sporadically occurring (community

-2-

acquired) type. However, until recently, no transmissible agent responsible for NANBH had been identified, and clinical diagnosis and identification of NANBH had been accomplished primarily by exclusion of other viral markers. Among the methods used to detect putative NANBH antigens and antibodies were agar-gel diffusion, counterimmunoelectrophoresis, immunofluorescence microscopy, immune electron microscopy, radioimmunoassay, and enzyme-linked immunosorbent assay. However, none of these assays proved to be sufficiently sensitive, specific, and reproducible to be used as a diagnostic test for NANBH.

In 1987, scientists at Chiron Corporation (the owner of the present application) identified the first nucleic acid definitively linked to blood-borne NANBH. See, e.g., EPO Pub. No. 318,216; Houghton et al., Science 244:359 (1989). These publications describe the cloning of an isolate from a new viral class, hepatitis C virus (HCV), the prototype isolate described therein being named "HCV1." HCV is a Flavi-like virus, with an RNA genome.

U.S. Patent Application Serial No. 456,637 (Houghton et al.), incorporated herein by reference, describes the preparation of various recombinant HCV polypeptides by expressing HCV cDNA and the screening of those polypeptides for immunological reactivity with sera from HCV patients. That limited screening showed that at least five of the polypeptides tested were very immunogenic; specifically, those identified as 5-1-1, C100, C33c, CA279a, and CA290a. Of these five polypeptides, 5-1-1 is located in the putative NS4 domain; C100 spans the putative NS3 and NS4 domains; C33c is located within the putative NS3 domain and CA279a and CA290a are located within the putative C domain. The screening also showed

35

SUBSTITUTE SHEET

-3-

that no single polypeptide tested was immunologically reactive with all sera. Thus, improved tests, which react with all or more samples from HCV positive individuals, are desirable.

5

Disclosure of the Invention

Applicants have carried out additional serological studies on HCV antigens that confirm that no single HCV polypeptide identified to date is immunologically reactive with all sera. This lack of a single polypeptide that is universally reactive with all sera from individuals with HCV may be due, inter alia, to strain-to-strain variation in HCV epitopes, variability in the humoral response from individual-to-individual and/or variation in serology with the state of the disease.

These additional studies have also enabled applicants to identify combinations of HCV antigens that provide more efficient detection of HCV antibodies than any single HCV polypeptide.

Accordingly, one aspect of this invention is a combination of HCV antigens comprising:

- (a) a first HCV antigen from the C domain; and
- (b) at least one additional HCV antigen selected from the group consisting of

- (i) an HCV antigen from the NS3 domain;
- (ii) an HCV antigen from the NS4 domain;
- (iii) an HCV antigen from the S domain;

and

- (iv) an HCV antigen from the NS5 domain.

In one embodiment, the combination of HCV antigens is in the form of a fusion protein comprised of the antigens. In an alternative embodiment, the combination of antigens is in the form of the individual antigens bound to a common solid matrix. In still another embodiment, the combination of antigens is in the form of a mixture of the individual antigens.

-4-

Another aspect of the invention is a method for detecting antibodies to HCV in a mammalian body component suspected of containing said antibodies comprising contacting said body component with the above-described
5 combination of HCV antigens under conditions that permit antibody-antigen reaction and detecting the presence of immune complexes of said antibodies and said antigens.

Another aspect of the invention is a method for detecting antibodies to HCV in a mammalian body component
10 suspected of containing said antibodies comprising contacting said body component with a panel of HCV antigens, simultaneously or sequentially, comprising
(a) a first HCV antigen from the C domain; and
(b) at least one additional HCV antigen
15 selected from the group consisting of
(i) an HCV antigen from the NS3 domain;
(ii) an HCV antigen from the NS4 domain;
(iii) an HCV antigen from the S domain;
and
20 (iv) an HCV antigen from the NS5 domain
under conditions that permit antibody-antigen reaction and detecting the presence of immune complexes of said antibodies and said antigens.

Another aspect of the invention is a kit for
25 carrying out an assay for detecting antibodies to HCV in a mammalian body component suspected of containing said antibodies comprising in packaged combination
(a) said combination of HCV antigens;
(b) standard control reagents; and
30 (c) instructions for carrying out the assay.

Brief Description of the Drawings

In the drawings:

Figure 1 is the nucleotide sequence of the cDNA
35 sense and anti-sense strand for the HCV polyprotein and the amino acid sequence encoded by the sense strand.

-5-

Figure 2 is a schematic of the amino acid sequence of Figure 1 showing the putative domains of the HCV polypeptide.

5 Modes for Carrying Out the Invention

Definitions

"HCV antigen" intends a polypeptide of at least about 5 amino acids, more usually at least about 8 to 10 amino acids that defines an epitope found in an isolate of HCV. Preferably, the epitope is unique to HCV. When an antigen is designated by an alphanumeric code, the epitope is from the HCV domain specified by the alphanumeric.

"Synthetic" as used to characterize an HCV antigen intends that the HCV antigen has either been isolated from native sources or man-made such as by chemical or recombinant synthesis.

"Domains" intends those segments of the HCV polyprotein shown in Figure 2 which generally correspond to the putative structural and nonstructural proteins of HCV. Domain designations generally follow the convention used to name Flaviviral proteins. The locations of the domains shown in Figure 2 are only approximate. The designations "NS" denotes "nonstructural" domains, while "S" denotes the envelope domain, and "C" denotes the nucleocapsid or core domain.

"Fusion polypeptide" intends a polypeptide in which the HCV antigen(s) are part of a single continuous chain of amino acids, which chain does not occur in nature. The HCV antigens may be connected directly to each other by peptide bonds or be separated by intervening amino acid sequences. The fusion polypeptides may also contain amino acid sequences exogenous to HCV.

35

-6-

"Common solid matrix" intends a solid body to which the individual HCV antigens or the fusion polypeptide comprised of HCV antigens are bound covalently or by noncovalent means such as hydrophobic adsorption.

5 "Mammalian body component" intends a fluid or tissue of a mammalian individual (e.g., a human) that commonly contains antibodies produced by the individual. Such components are known in the art and include, without limitation, blood, plasma, serum, spinal fluid, lymph
10 fluid, secretions of the respiratory, intestinal or genitourinary tracts, tears, saliva, milk, white blood cells, and myelomas.

"Immunologically reactive" means that the antigen in question will react specifically with anti-HCV
15 antibody commonly present in a significant proportion of sera from individuals infected with HCV.

"Immune complex" intends the combination or aggregate formed when an antibody binds to an epitope on an antigen.

20

Combinations of HCV Antigens

Figure 2 shows the putative domains of the HCV polyprotein. The domains from which the antigens used in the combinations derive are: C, S (or E), NS3, NS4, and
25 NS5. The C domain is believed to define the nucleocapsid protein of HCV. It extends from the N-terminal of the polyprotein to approximately amino acid 120 of Figure 1. The S domain is believed to define the virion envelope protein, and possibly the matrix (M) protein, and is
30 believed to extend from approximately amino acid 120 to amino acid 400 of Figure 1. The NS3 domain extends from approximately amino acid 1050 to amino acid 1640 and is believed to constitute the viral protease. The NS4 domain
35 extends from the terminus of NS3 to approximately amino acid 2000. The function of the NS4 protein is not known at this time. Finally, the NS5 domain extends from about

-7-

amino acid 2000 to the end of the polyprotein and is believed to define the viral polymerase.

The sequence shown in Figure 1 is the sequence of the HCV1 isolate. It is expected that the sequences of other strains of the blood-borne HCV may differ from the sequence of Figure 1, particularly in the envelope (S) and nucleocapsid (C) domains. The use of HCV antigens having such differing sequences is intended to be within the scope of the present invention; provided, however, that the variation does not significantly degrade the immunological reactivity of the antigen to sera from persons infected with HCV.

In general, the HCV antigens will comprise entire or truncated domains, the domain fragments being readily screened for antigenicity by those skilled in the art. The individual HCV antigens used in the combination will preferably comprise the immunodominant portion (i.e., the portion primarily responsible for the immunological reactivity of the polypeptide) of the stated domain. In the case of the C domain it is preferred that the C domain antigen comprise a majority of the entire sequence of the domain. The antigen designated C22 (see Example 4, *infra*), is particularly preferred. The S domain antigen preferably includes the hydrophobic subdomain at the N-terminal end of the domain. This hydrophobic subdomain extends from approximately amino acid 199 to amino acid 328 of Figure 1. The HCV antigen designated S2 (see Example 3, *infra*), is particularly preferred. Sequence downstream of the hydrophobic subdomain may be included in the S domain antigen if desired.

A preferred NS3 domain antigen is the antigen designated C33c. That antigen includes amino acids 1192 to 1457 of Figure 1. A preferred NS4 antigen is C100 which comprises amino acids 1569 to 1931 of Figure 1. A preferred NS5 antigen comprises amino acids 2054 to 2464 of Figure 1.

-8-

The HCV antigen may be in the form of a polypeptide composed entirely of HCV amino acid sequence or it may contain sequence exogenous to HCV (i.e., it may be in the form of a fusion protein that includes exogenous sequence). In the case of recombinantly produced HCV antigen, producing the antigen as a fusion protein such as with SOD, alpha-factor or ubiquitin (see commonly owned U.S. Pat. No. 4,751,180, U.S. Pat. No. 4,870,008 and U.S. Pat. Application Serial. No. 390,599, filed 7 August 1989, the disclosures of which are incorporated herein, which describe expression of SOD, alpha-factor and ubiquitin fusion proteins) may increase the level of expression and/or increase the water solubility of the antigen. Fusion proteins such as the alpha-factor and ubiquitin fusion are processed by the expression host to remove the heterologous sequence. Alpha-factor is a secretion system, however, while ubiquitin fusions remain in the cytoplasm.

Further, the combination of antigens may be produced as a fusion protein. For instance, a continuous fragment of DNA encoding C22 and C33c may be constructed, cloned into an expression vector and used to express a fusion protein of C22 and C33c. In a similar manner fusion proteins of C22 and C100; C22 and S2; C22 and an NS5 antigen; C22, C33c, and S2; C22, C100 and S2, and C22, C33c, C100, and S2 may be made. Alternative fragments from the exemplified domain may also be used.

Preparation of HCV Antigens

The HCV antigens of the invention are preferably produced recombinantly or by known solid phase chemical synthesis. They may, however, also be isolated from dissociated HCV or HCV particles using affinity chromatography techniques employing antibodies to the antigens.

-9-

When produced by recombinant techniques, standard procedures for constructing DNA encoding the antigen, cloning that DNA into expression vectors, transforming host cells such as bacteria, yeast, insect, or mammalian cells, and expressing such DNA to produce the antigen may be employed. As indicated previously, it may be desirable to express the antigen as a fusion protein to enhance expression, facilitate purification, or enhance solubility. Examples of specific procedures for producing representative HCV antigens are described in the Examples, infra, and in parent application Serial No. 456,637.

Formulation of Antigens for Use in Immunoassay

The HCV antigens may be combined by producing them in the form of a fusion protein composed of two or more of the antigens, by immobilizing them individually on a common solid matrix, or by physically mixing them. Fusion proteins of the antigen may also be immobilized on (bound to) a solid matrix. Methods and means for covalently or noncovalently binding proteins to solid matrices are known in the art. The nature of the solid surface will vary depending upon the assay format. For assays carried out in microtiter wells, the solid surface will be the wall of the well or cup. For assays using beads, the solid surface will be the surface of the bead. In assays using a dipstick (i.e., a solid body made from a porous or fibrous material such as fabric or paper) the surface will be the surface of the material from which the dipstick is made. In agglutination assays the solid surface may be the surface of latex or gelatin particles. When individual antigens are bound to the matrix they may be distributed homogeneously on the surface or distributed thereon in a pattern, such as bands so that a pattern of antigen binding may be discerned.

Simple mixtures of the antigens comprise the antigens in any suitable solvent or dispersing medium.

-10-

Assay Formats Using Combinations of Antigens

The HCV antigens may be employed in virtually any assay format that employs a known antigen to detect antibodies. A common feature of all of these assays is that the antigen is contacted with the body component suspected of containing HCV antibodies under conditions that permit the antigen to bind to any such antibody present in the component. Such conditions will typically be physiologic temperature, pH and ionic strength using an excess of antigen. The incubation of the antigen with the specimen is followed by detection of immune complexes comprised of the antigen.

Design of the immunoassays is subject to a great deal of variation, and many formats are known in the art. Protocols may, for example, use solid supports, or immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, enzymatic, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the immune complex are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

The immunoassay may be, without limitation, in a heterogeneous or in a homogeneous format, and of a standard or competitive type. In a heterogeneous format, the polypeptide is typically bound to a solid matrix or support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used are nitrocellulose (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates, polyvinylidene fluoride (known as Immulon[®]), diazotized paper, nylon membranes, activated beads, and Protein A beads. For example, Dynatech

-11-

Immulon™ 1 or Immulon™ 2 microtiter plates or 0.25 inch polystyrene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic polypeptides is typically washed after
5 separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are known in the art.

In a homogeneous format, the test sample is incubated with the combination of antigens in solution.
10 For example, it may be under conditions that will precipitate any antigen-antibody complexes which are formed. Both standard and competitive formats for these assays are known in the art.

In a standard format, the amount of HCV antibodies forming the antibody-antigen complex is directly
15 monitored. This may be accomplished by determining whether labeled anti-xenogenic (e.g., anti-human) antibodies which recognize an epitope on anti-HCV antibodies will bind due to complex formation. In a competitive format,
20 the amount of HCV antibodies in the sample is deduced by monitoring the competitive effect on the binding of a known amount of labeled antibody (or other competing ligand) in the complex.

Complexes formed comprising anti-HCV antibody
25 (or, in the case of competitive assays, the amount of competing antibody) are detected by any of a number of known techniques, depending on the format. For example, unlabeled HCV antibodies in the complex may be detected using a conjugate of antixenogeneic Ig complexed with a
30 label, (e.g., an enzyme label).

In an immunoprecipitation or agglutination assay format the reaction between the HCV antigens and the antibody forms a network that precipitates from the solution or suspension and forms a visible layer or film of
35 precipitate. If no anti-HCV antibody is present in the test specimen, no visible precipitate is formed.

-12-

The HCV antigens will typically be packaged in the form of a kit for use in these immunoassays. The kit will normally contain in separate containers the combination of antigens (either already bound to a solid matrix or separate with reagents for binding them to the matrix), control antibody formulations (positive and/or negative), labeled antibody when the assay format requires same and signal generating reagents (e.g., enzyme substrate) if the label does not generate a signal directly. Instructions (e.g., written, tape, VCR, CD-ROM, etc.) for carrying out the assay usually will be included in the kit.

The following examples are intended to illustrate the invention and are not intended to limit the invention in any manner.

Example 1: Synthesis of HCV Antigen C33c

HCV antigen C33c contains a sequence from the NS3 domain. Specifically, it includes amino acids 1192-1457 of Figure 1. This antigen was produced in bacteria as a fusion protein with human superoxide dismutase (SOD) as follows. The vector pSODcfl (Steiner et al. (1986), J. Virol. 58:9) was digested to completion with EcoRI and BamHI and the resulting EcoRI, BamHI fragment was ligated to the following linker to form pcflEF:

GATC CTG GAA TTC TGA TAA
GAC CTT AAG ACT ATT TTA A

A cDNA clone encoding amino acids 1192-1457 and having EcoRI ends was inserted into pcflEF to form pcflEF/C33c. This expression construct was transformed into D1210 E. coli cells.

The transformants were used to express a fusion protein comprised of SOD at the N-terminus and in-frame C33c HCV antigen at the C-terminus. Expression was ac-

-13-

complished by inoculating 1500 ml of Luria broth containing ampicillin (100 micrograms/ml) with 15 ml of an overnight culture of the transformants. The cells were grown to an O.D. of 0.3, IPTG was added to yield a final concentration of 2 mM, and growth continued until the
5 cells attained a density of 1 O.D., at which time they were harvested by centrifugation at 3,000 x g at 4°C for 20 minutes. The packed cells can be stored at -80°C for several months.

10 In order to purify the SOD-C33c polypeptide the bacterial cells in which the polypeptide was expressed were subjected to osmotic shock and mechanical disruption, the insoluble fraction containing SOD-C33c was isolated and subjected to differential extraction with an alkaline-
15 NaCl solution, and the fusion polypeptide in the extract purified by chromatography on columns of S-Sepharose and Q-Sepharose.

The crude extract resulting from osmotic shock and mechanical disruption was prepared by the following
20 procedure. One gram of the packed cells were suspended in 10 ml of a solution containing 0.02 M Tris HCl, pH 7.5, 10 mM EDTA, 20% sucrose, and incubated for 10 minutes on ice. The cells were then pelleted by centrifugation at 4,000 x g for 15 min at 4°C. After the supernatant was removed,
25 the cell pellets were resuspended in 10 ml of Buffer A1 (0.01M Tris HCl, pH 7.5, 1 mM EDTA, 14 mM beta-mercaptoethanol [BME]), and incubated on ice for 10 minutes. The cells were again pelleted at 4,000 x g for 15 minutes at 4°C. After removal of the clear supernatant
30 (periplasmic fraction I), the cell pellets were resuspended in Buffer A1, incubated on ice for 10 minutes, and again centrifuged at 4,000 x g for 15 minutes at 4°C. The clear supernatant (periplasmic fraction II) was removed, and the cell pellet resuspended in 5 ml of Buffer
35 A2 (0.02 M Tris HCl, pH 7.5, 14 mM BME, 1 mM EDTA, 1 mM PMSF). In order to disrupt the cells, the suspension (5

-14-

- ml) and 7.5 ml of Dyno-mill lead-free acid washed glass beads (0.10-0.15 mm diameter) (obtained from Glen-Mills, Inc.) were placed in a Falcon tube, and vortexed at top speed for two minutes, followed by cooling for at least 2 min on ice; the vortexing-cooling procedure was repeated another four times. After vortexing, the slurry was filtered through a scintered glass funnel using low suction; the glass beads were washed two times with Buffer A2, and the filtrate and washes combined.
- 10 The insoluble fraction of the crude extract was collected by centrifugation at 20,000 x g for 15 min at 4°C, washed twice with 10 ml Buffer A2, and resuspended in 5 ml of MILLI-Q water.
- 15 A fraction containing SOD-C33c was isolated from the insoluble material by adding to the suspension NaOH (2 M) and NaCl (2 M) to yield a final concentration of 20 mM each, vortexing the mixture for 1 minute, centrifuging it 20,000 x g for 20 min at 4°C, and retaining the supernatant.
- 20 In order to purify SOD-C33c on S-Sepharose, the supernatant fraction was adjusted to a final concentration of 6M urea, 0.05M Tris HCl, pH 7.5, 14 mM BME, 1 mM EDTA. This fraction was then applied to a column of S-Sepharose Fast Flow (1.5 x 10 cm) which had been equilibrated with
- 25 Buffer B (0.05M Tris HCl, pH 7.5, 14 mM BME, 1 mM EDTA). After application, the column was washed with two column volumes of Buffer B. The flow through and wash fractions were collected. The flow rate of application and wash, was 1 ml/min; and collected fractions were 1 ml. In order
- 30 to identify fractions containing SOD-C33c, aliquots of the fractions were analyzed by electrophoresis on 10% polyacrylamide gels containing SDS followed by staining with Coomassie blue. The fractions are also analyzable by Western blots using an antibody directed against SOD.
- 35 Fractions containing SOD-C33c were pooled.

SUBSTITUTE SHEET

-15-

Further purification of SOD-C33c was on a Q-Sepharose column (1.5 x 5 cm) which was equilibrated with Buffer B. The pooled fractions containing SOD-C33c obtained from chromatography on S-Sepharose was applied to the column. The column was then washed with Buffer B, and eluted with 60 ml of a gradient of 0.0 to 0.4 M NaCl in Buffer B. The flow rate for application, wash, and elution was 1 ml/min; collected fractions were 1 ml. All fractions from the Q-Sepharose column were analyzed as described for the S-Sepharose column. The peak of SOD-C33c eluted from the column at about 0.2 M NaCl.

The SOD-C33c obtained from the Q-Sepharose column was greater than about 90% pure, as judged by analysis on the polyacrylamide SDS gels and immunoblot using a monoclonal antibody directed against human SOD.

Example 2: Synthesis of HCV Antigen C100

HCV antigen C100 contains sequences from the NS3 and NS4 domains. Specifically, it includes amino acids 1569-1931 of Figure 1. This antigen was produced in yeast. A cDNA fragment of a 1270 bp encoding the above amino acids and heaving EcoRI termini was prepared.

The construction of a yeast expression vector in which this fragment was fused directly to the S. cerevisiae ADH2/GAP promoter was accomplished by a protocol which included amplification of the C100 sequence using a PCR method, followed by ligation of the amplified sequence into a cloning vector. After cloning, the C100 sequence was excised, and with a sequence which contained the ADH2/GAP promoter, was ligated to a large fragment of a yeast vector to yield a yeast expression vector.

The PCR amplification of C100 was performed using as template the vector pS3-56_{C100m}, which had been linearized by digestion with SalI. pS3-56, which is a pBR322 derivative, contains an expression cassette which is comprised of the ADH2/GAPDH hybrid yeast promoter

-16-

upstream of the human superoxide dismutase gene, and a downstream alpha factor transcription terminator.

The oligonucleotide primers used for the amplification were designed to facilitate cloning into the expression vector, and to introduce a translation termination codon. Specifically, novel 5'-HindIII and 3'-SalI sites were generated with the PCR oligonucleotides. The oligonucleotide containing the SalI site also encodes the double termination codons, TAA-and TGA. The oligonucleotide containing the HindIII site also contains an untranslated leader sequence derived from the pgap63 gene, situated immediately upstream of the AUG codon. The pEco63GAPDH gene is described by Holland and Holland (1980) and by Kniskern et al. (1986). The PCR primer sequences used for the direct expression of C100m were:

5' GAG TGC TCA AGC TTC AAA ACA AAA TGG CTC
ACT TTC TAT CCC AGA CAA AGC AGA GT 3'

20

and

5' GAG TGC TCG TCG ACT CAT TAG GGG GAA
ACA TGG TTC CCC CGG GAG GCG AA 3'.

Amplification by PCR, utilizing the primers, and template, was with a Cetus-Perkin-Elmer PCR kit, and was performed according to the manufacturer's directions. The PCR conditions were 29 cycles of 94°C for a minute, 37°C for 2 minutes, 72°C for 3 minutes; and the final incubation was at 72°C for 10 minutes. The DNA can be stored at 4°C or -20°C overnight.

After amplification, the PCR products were digested with HindIII and SalI. The major product of 1.1 kb was purified by electrophoresis on a gel, and the eluted purified product was ligated with a large SalI-HindIII fragment of pBR322. In order to isolate correct

-17-

recombinants, competent HB101 cells were transformed with the recombinant vectors, and after cloning, the desired recombinants were identified on the basis of the predicted size of HindIII-SalI fragments excised from the clones.

- 5 One of the clones which contained the a HindIII-SalI fragment of the correct size was named pBR322/C100⁻d. Confirmation that this clone contained amplified C100 was by direct sequence analysis of the HindIII-SalI fragment.

- The expression vector containing C100 was
10 constructed by ligating the HindIII-SalI fragment from pBR322/C100⁻d to a 13.1 kb BamHI-SalI fragment of pBS24.1, and a 1369 bp BamHI-HindIII fragment containing the ADH2/GAP promoter. (The latter fragment is described in EPO 164,556). The pBS24.1 vector is described in commonly
15 owned U.S.S.N. 382,805 filed 19 July 1989. The ADH2/GAP promoter fragment was obtained by digestion of the vector pPGAP/AG/HindIII with HindIII and BamHI, followed by purification of the 1369 bp fragment on a gel.

- Competent HB101 cells were transformed with the
20 recombinant vectors; and correct recombinants were identified by the generation of a 2464 bp fragment and a 13.1 kb fragment generated by BamHI and SalI digestion of the cloned vectors. One of the cloned correct recombinant vectors was named pC100⁻d#3.

- 25 In order to express C100, competent cells of Saccharomyces cerevisiae strain AB122 (MATa leu2 ura3-53 prb 1-1122 pep4-3 prc1-407[cir-0]) were transformed with the expression vector pC100⁻d#3. The transformed cells were plated on URA-sorbitol, and individual transformants
30 were then streaked on Leu⁻ plates.

- Individual clones were cultured in Leu⁻, ura⁻ medium with 2% glucose at 30°C for 24-36 hours. One liter of Yeast Extract Peptone Medium (YEP) containing 2% glucose was inoculated with 10 ml of the overnight
35 culture, and the resulting culture was grown at 30°C at an agitation rate of 400 rpm and an aeration rate of 1 L of

-18-

air per 1 L of medium per minute (i.e., 1vvm) for 48 hours. The pH of the medium was not controlled. The culture was grown in a BioFlo II fermentor manufactured by New Brunswick Science Corp. Following fermentation, the
5 cells were isolated and analyzed for C100 expression.

Analysis for expressed C100 polypeptide by the transformed cells was performed on total cell lysates and crude extracts prepared from single yeast colonies obtained from the Leu⁻ plates. The cell lysates and crude
10 extracts were analyzed by electrophoresis on SDS polyacrylamide gels, and by Western blots. The Western blots were probed with rabbit polyclonal antibodies directed against the SOD-C100 polypeptide expressed in yeast. The expected size of the C100 polypeptide is 364
15 amino acids. By gel analysis the expressed polypeptide has a MW_r of 39.9K.

Both analytical methods demonstrated that the expressed C100 polypeptide was present in total cell lysates, but was absent from crude extracts. These
20 results suggest that the expressed C100 polypeptide may be insoluble.

Example 3: Expression of HCV Antigen S2

HCV antigen S2 contains a sequence from the
25 hydrophobic N-terminus of the S domain. It includes amino acids 199-328 of Figure 1.

The protocol for the construction of the expression vector encoding the S2 polypeptide and for its expression in yeast was analogous to that used for the
30 expression of the C100 polypeptide, described in Example 2.

The template for the PCR reaction was the vector pBR322/Pil4a, which had been linearized by digestion with HindIII. Pil4a is a cDNA clone that encodes amino acids
35 199-328.

-19-

The oligonucleotides used as primers for the amplification by PCR of the S2 encoding sequence were the following.

- 5 For the 5'-region of the S2 sequence:

5' GAG TGC TCA AGC TTC AAA ACA AAA TGG GGC TCT
ACC ACG TCA CCA ATG ATT GCC CTA AC 3';

and -

10

for the 3'-region of the S2 sequence:

5' GAG TGC TCG TCG ACT CAT TAA GGG GAC CAG TTC
ATC ATC ATA TCC CAT GCC AT 3'.

- 15 The primer for the 5'-region introduces a HindIII site and an ATG start codon into the amplified product. The primer for the 3'-region introduces translation stop codons and a SalI site into the amplified product.

- 20 The PCR conditions were 29 cycles of 94°C for a minute, 37°C for 2 minutes, 72°C for 3 minutes, and the final incubation was at 72°C for 10 minutes.

- The main product of the PCR reaction was a 413 bp fragment, which was gel purified. The purified fragment was ligated to the large fragment obtained from pBR322 digested with HindIII and SalI fragment, yielding the plasmid pBR322/S2d.

- Ligation of the 413 bp HindIII-SalI S2 fragment with the 1.36 kb BamHI-HindIII fragment containing the ADH2/GAP promoter, and with the large BamHI-SalI fragment of the yeast vector pBS24.1 yielded recombinant vectors, which were cloned. Correct recombinant vectors were identified by the presence of a 1.77 kb fragment after digestion with BamHI and SalI. An expression vector constructed from the amplified sequence, and containing the sequence encoding S2 fused directly to the ADH2/GAP promoter is identified as pS2d#9.

-20-

Example 4: Synthesis of HCV C Antigen

HCV antigen C22 is from the C domain. It comprises amino acids 1-122 of Figure 1.

5 The protocol for the construction of the expression vector encoding the C polypeptide and for its expression in yeast was analogous to that used for the expression of the C100 polypeptide, described supra, except for the following.

10 The template for the PCR reaction was pBR322/Ag30a which had been linearized with HindIII. Ag30 is a cDNA clone that encodes amino acids 1-122. The oligonucleotides used as primers for the amplification by PCR of the C encoding sequence were the following.

15 For the 5'-region of the C sequence:

5' GAG TGC AGC TTC AAA ACA AAA TGA GCA CGA
ATC CTA AAC CTC AAA AAA AAA AC 3',

20 and

for the 3'-region of the C sequence:

5' GAG TGC TCG TCG ACT CAT TAA CCC AAA TTG CGC
GAC CTA CGC CGG GGG TCT GT 3'.

25 The primer for the 5'-region introduces a HindIII site into the amplified product, and the primer for the 3'-region introduces translation stop codons and a Sall site. The PCR was run for 29 cycles of 94°C for a minute, 37°C
30 for 2 minutes, 72°C for 3 minutes, and the final incubation was at 72°C for 10 minutes.

The major product of PCR amplification is a 381 bp polynucleotide. Ligation of this fragment with the Sall-HindIII large Sall-HindIII fragment of pBR322 yielded
35 the plasmid pBR322/C2.

-21-

Ligation of the 381 bp HindIII-SalI C coding fragment excised from pBR322/C2 with the 1.36 kb BamHI-HindIII fragment containing the ADH2/GAP promoter, and with the large BamHI-SalI fragment of the yeast vector pBS24.1 yielded recombinant vectors, which were cloned. Correct recombinant vectors were identified by the presence of a 1.74 kb fragment after digestion with BamHI and SalI. An expression vector constructed from the amplified sequence, and containing the sequence encoding C fused directly to the ADH2/GAP promoter is identified as pC22.

Analysis for expressed C polypeptide by the transformed cells was performed on total cell lysates and crude extracts prepared from single yeast colonies obtained from the Leu⁻ plates. The cell lysates and crude extracts were analyzed by electrophoresis on SDS polyacrylamide gels. The C polypeptide is expected to have 122 amino acids and by gel analysis the expressed polypeptide has a MW_r of approximately 13.6 Kd.

20 Example 5: Synthesis of NS5 Polypeptide

This polypeptide contains sequence from the N-terminus of the NS5 domain. Specifically it includes amino acids 2054 to 2464 of Figure 1. The protocol for the construction of the expression vector encoding the NS5 polypeptide and for its expression were analogous to that used for the expression of C33c (see Example 1).

Example 6: Radioimmunoassay (RIA) for Antibodies to HCV

The HCV antigens of Examples 1-5 were tested in an RIA format for their ability to detect antibodies to HCV in the serum of individuals clinically diagnosed as having HCV (Non-A, Non-B) and in serum from blood given by paid blood donors.

The RIA was based upon the procedure of Tsu and Herzenberg (1980) in SELECTED METHODS IN CELLULAR IMMUNOLOGY (W.H. Freeman & Co.), pp. 373-391. Generally,

-22-

microtiter plates (Immulon 2, Removawell strips) are coated with purified HCV antigen. The coated plates are incubated with the serum samples or appropriate controls. During incubation, antibody, if present, is immunologically bound to the solid-phase antigen. After removal of the unbound material and washing of the microtiter plates, complexes of human antibody-NANBV antigen are detected by incubation with ^{125}I -labeled sheep anti-human immunoglobulin. Unbound labeled antibody is removed by aspiration, and the plates are washed. The radioactivity in individual wells is determined; the amount of bound human anti-HCV antibody is proportional to the radioactivity in the well.

Specifically, one hundred microliter aliquots containing 0.1 to 0.5 micrograms of the HCV antigen in 0.125 M Na borate buffer, pH 8.3, 0.075 M NaCl (BBS) was added to each well of a microtiter plate (Dynatech Immulon 2 Removawell Strips). The plate was incubated at 4°C overnight in a humid chamber, after which, the antigen solution was removed and the wells washed 3 times with BBS containing 0.02% Triton X-100 (BBST). To prevent non-specific binding, the wells were coated with bovine serum albumin (BSA) by addition of 100 microliters of a 5 mg/ml solution of BSA in BBS followed by incubation at room temperature for 1 hour; after this incubation the BSA solution was removed. The antigens in the coated wells were reacted with serum by adding 100 microliters of serum samples diluted 1:100 in 0.01M Na phosphate buffer, pH 7.2, 0.15 M NaCl (PBS) containing 10 mg/ml BSA, and incubating the serum containing wells for 1 hr at 37°C . After incubation, the serum samples were removed by aspiration, and the wells were washed 5 times with BBST. Antibody bound to the antigen was determined by the binding of ^{125}I -labeled $\text{F}'(\text{ab})_2$ sheep anti-human IgG to the coated wells. Aliquots of 100 microliters of the labeled probe (specific activity 5-20 microcuries/microgram) were

SUBSTITUTE SHEET

-23-

added to each well, and the plates were incubated at 37°C for 1 hour, followed by removal of excess probe by aspiration, and 5 washes with BBST. The amount of radioactivity bound in each well was determined by counting in a counter which detects gamma radiation.

Table 1 below presents the results of the tests on the serum from individuals diagnosed as having HCV.

Table 1

10

	<u>INDIVIDUAL</u>	<u>ANTIGEN</u>				
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u>	<u>NS5</u>
	CVH IVDA	P	P	P(+++)	P	P
	CVH IVDA	P	P	P(+)	P	P
15	CVH IVDA	P	P	P(+)	P	P
	CVH NOS	P	P	P	P	P
	AVH NOS HS	N	N	N	N	N
	AVH NOS HS	P	N	N	N	N
	AVH NOS HS	P	N	N	N	N
20	AVH NOS HS	P/N	N	N	N	N
	AVH PTVH	N	N	N	P/N	N
	AVH NOS HS	N	N	N	N	N
	AVH NOS	N	N	N	N	P
	AVH PTVH	N	N	N	N	N
25	AVH IVDA	N	P	N	P	P
	AVH PTVH	P	P/N	N	N	P
	AVH NOS	N	P	N	N	N
	AVH IVDA	N	P	N	P	P
	AVH NOS HS	P/N	N	N	N	N
30	AVH PTVH	N	N	N	N	N
	CVH IVDA	P	P	P	P	P
	CVH IVDA	P	P	P	P	P
	AVH NOS HS	N	N	N	N	N
	CVH PTVH	P	P	N	N	N
35	AVH PTVH	P	N	P(+)	P(+++)	N
	CVH PTVH	N	P	P	P	P
	CVH NOS HS	P	P	P	P	N
	--- NOS	N	P	P/N	P	P

-24-

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>			
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u> <u>NS5</u>
	CVH IVDA	N	N	N	P N
	AVH IVDA	P	P	P	P P
5	AVH IVDA	P	P	P	P P
	CVH IVDA	P	P	P	P P
	AVH IVDA	P/N	P	N	P P
	AVH IVDA	N	P	P	P N
	CVH PTVH	P	P/N	N	N N
10	CVH NOS	N	N	N	N N
	CVH NOS	N	N	N	N N
	CVH IVDA	P	P	P	P P
	AVH IVDA	P	P	P	P P
	CVH PTVH	P	P	P	P P
15	AVH PTVH?	N	P	P	P P
	AVH IVDA	N	P	N	P N
	AVH NOS	N	N	N	N N
	AVH NOS	N	N	N	N N
	CVH NOS	N	P	N	N P
20	CVH NOS	P	P	N	N N
	CVH NOS HS	P	P	P	P P
	CVH PTVH	P	P	N	P P
	AVH nurse	P	P	N	N N
	AVH IVDA	P	P	P	P N
25	AVH IVDA	N	P	P(+)	P(+++) N
	AVH nurse	P/N	P	N	N N
	AVH PTVH	P/N	P	P	N P
	AVH NOS	N	P/N	N	N P
	AVH NOS	N	P	N	P N
30	AVH PTVH	P	P/N	N	N N
	AVH PTVH	N	P	N	P P
	AVH PTVH	P	P	P	P P
	AVH PTVH	N	P	N	N P
	CVH PTVH	P/N	P	P(+)	P(+++) N
35	AVH PTVH	N	P/N	P(+)	P(+++) P

SUBSTITUTE SHEET

-25-

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>			
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u> <u>NS5</u>
	AVH PTVH	P	(?)	P	N P
	CVH PTVH	N	P	N	P P
5	CVH PTVH	N	P	P	P P
	CVH PTVH	N	N	N	N N
	AVH NOS	N	N	N	N N
	AVH nurse	P	P	N	N N
	CVH PTVH	N	P	N	N P
10	AVH IVDA	N	P	N	P/N N
	CVH PTVH	P	P	P(+)	P(+++) P
	AVH NOS	P	P	N	N N
	AVH NOS	P/N	P	N	N P
	AVH PTVH	P/N	P	P	P P
15	AVH NOS	N	P	P	P P/N
	AVH IVDA	N	P	N	N P
	AVH NOS	N	P/N	N	N N
	AVH NOS	P	P	N	N P
	AVH PTVH	N	P	P	P P
20	crypto	P	P	P	P P
	CVH NOS	N	P	P	P P
	CVH NOS	N	N	N	N N
	AVH PTVH	N	P	P(+)	P(+++) N
	AVH PTVH	N	P/N	P(+)	P(+++) P
25	AVH PTVH	N	P/N	P(+)	P(+++) P
	CVH IVDA	P	P	P	P P
	CVH IVDA	P	P	P	P P
	CVH IVDA	P	P	P	P P
	CVH IVDA	P	P	P	P P
30	AVH NOS	N	P	N	N N
	CVH IVDA	P	P	P	P P/N
	AVH IVDA	P	P	P	P N
	AVH NOS	P	P	N	N N
	AVH NOS	P	P	N	N N
35	CVH PTVH	P	P	N	N P/N

-26-

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>			
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u> <u>NS5</u>
	AVH PTVH	N	P	N	P P
	AVH NOS	N	N	N	N N
5	AVH NOS	N	P	N	N N
	AVH NOS	P	N	N	N N
	CVH NOS	N	N	N	N N
	AVH NOS	N	P/N	N	N N
	AVH IVDA	N	P	P	P P
10	crypto	N	P	N	N P/N
	crypto	P	P	P/N	P P
	AVH IVDA	N	N	P	P N
	AVH IVDA	N	P	P	P N
	AVH NOS	N	N	N	N N
15	AVH NOS	N	N	N	N N
	CVH IVDA	P	P	P	P P
	CVH PTVH	N	N	N	N N
	CVH PTVH	P	P	P(+)	P(+++) P
	CVH PTVH	P	P	P(+)	P(+++) P
20	CVH NOS	P/N	N	N	N N
	CVH NOS	N	N	N	N N
	CVH PTVH	P	P	P	P P
	CVH PTVH	P	P	P	P P
	CVH PTVH	P	P	P	P P
25	AVH IVDA	N	P	P	P P
	CVH NOS	N	N	N	N N
	CVH NOS	N	N	N	N N
	CVH PTVH	P	P	P	P P
	AVH NOS	P	P	N	N P/N
30	AVH NOS	N	P/N	N	N N
	CVH PTVH	P	F	N	N P
	CVH NOS	N	P/N	N	N N
	AVH NOS	N	F	N	N N
	AVH NOS	N	F	N	N N
35	CVH PTVH	N	P	N	N N

SUBSTITUTE SHEET

-27-

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>			
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u> <u>NS5</u>
	AVH IVDA	N	P	N	P P
	AVH NOS	P	N	N	N N
5	CVH NOS	N	N	N	N N
	CVH NOS	N	N	N	N N
	CVH IVDA	P	P	P	P P
	CVH IVDA	P/N	P	P	P P
	CVH IVDA	P	P	P	P P
10	CVH IVDA	N	P	P	P P
	AVH NOS	N	P	N	N N
	CVH IVDA	N	P	N	N P
	CVH IVDA	N	P	N	N P
	AVH PTVH	P	P	N	P P
15	AVH PTVH	P	P	N	P P
	CVH NOS	N	P/N	N	N P/N
	CVH NOS	N	P	N	N N
	CVH NOS	N	N	N	N N
	CVH PTVH	P	P	P	P P
20	CVH PTVH	P	P	P	P P
	CVH PTVH	P	P	P	P P
	AVH IVDA	N	P	N	N P
	AVH IVDA	N	P	P(++)	P(+) P
	CVH PTVH	P	P	P	P P
25	AVH PTVH	N	P	P	P P
	CVH PTVH?	N	P	P	P P
	CVH PTVH?	P/N	P	P	P P
	CVH NOS HS	P	P	N	N N
	CVH IVDA	P	P	P	P N
30	CVH PTVH	N	P	P	P P
	CVH PTVH	P	P	P	P P/N
	CVH NOS	P	P	P	P P
	CVH IVDA	P	F	F	P P
	CVH PTVH	P	P	P	P N
35	CVH PTVH	P	P	P	P P

-28-

<u>INDIVIDUAL</u>		<u>ANTIGEN</u>			
		<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u> <u>NS5</u>
	CVH NOS	N	N	N	N P/N
	CVH NOS	N	P/N	N	N P/N
5	CVH PTVH	P	P	P	P P
	CVH NOS	N	P	N	N N
	CVH NOS	N	N	N	N N
	CVH NOS	P	P	N	N P/N
	CVH NOS	N	N	N	N N
10	CVH NOS HS	P	P	P	P P
	CVH NOS HS	P	P	P	P P
	CVH PTVH	N	N	N	N N
	AVH PTVH	N	P	P	P P
	AVH NOS			-	-
15	CVH PTVH	N	P	P/N	P(+++) N
	crypto	P	P	P	P P
	crypto	P	P	P	P P
	crypto	N	P	N	N N
	crypto	N	P	P	P P
20	CVH PTVH	P	P	P	P P
	crypto	N	N	N	N N
	crypto	N	P	N	N P/N
	crypto	N	P	N	N P
	crypto	P	P	P	P P
25	crypto	N	P	N	P N
	crypto			-	-
	crypto			-	-
	CVH NOS			-	-
30	AVH-IVDA	N	P	N	P(+) P

35

SUBSTITUTE SHEET

-29-

INDIVIDUALANTIGEN

	<u>S2</u>	<u>C22</u>	<u>C100</u>	<u>C33c</u>	<u>NS5</u>
AVH-IVDA	N	P/N	N	P(++)	N

5

AVH = acute viral hepatitis

CVH = chronic viral hepatitis

PTVH = post-transfusion viral hepatitis

IVDA = intravenous drug abuser

10

crypto = cryptogenic hepatitis

NOS = non-obvious source

P = positive

N = negative

15

Per these results, no single antigen reacted with all sera. C22 and C33c were the most reactive and S2 reacted with some sera from some putative acute HCV cases with which no other antigen reacted. Based on these results, the combination of two antigens that would provide the greatest range of detection is C22 and C33c. If one wished to detect a maximum of acute infections, S2 would be included in the combination.

20

Table 2 below presents the results of the testing on the paid blood donors.

25

Table 2

		Antigens				
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
30	1	N	N	N	N	N
	2	N	N	N	N	N
	3	P	P	F	P	P
	4	N	N	N	N	N
	5	N	N	N	N	N
35	6	N	N	N	N	N
	7	N	N	N	N	N
	8	N	N	N	N	N

-30-

Antigens						
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
	9	N	N	N	N	N
	10	N	N	N	N	N
5	11	N	N	N	N	N
	12	N	N	N	N	N
	13	N	N	N	N	N
	14	N	N	N	N	N
	15	N	N	N	N	N
10	16	N	N	N	N	N
	17	N	N	N	N	N
	18	P	P	P	P	P
	19	P	P	N	P	P
	20	P	P	N	P	P
15	21	N	N	N	N	N
	22	N	P	P	N	P
	23	P	P	P	P	P
	24	N	N	N	N	N
	25	N	N	N	N	N
20	26	N	N	N	N	N
	27	N	N	N	N	N
	28	N	N	N	N	N
	29	N	N	N	N	N
	30	N	N	N	N	N
25	31	P	P	P	N	P
	32	N	N	N	N	N
	33	N	N	N	N	N
	34	N	N	N	N	P
	35	N	N	P	N	P
30	36	N	N	N	N	N
	37	N	N	N	N	N
	38	N	N	N	N	N
	39	N	N	N	N	N
	40	N	N	N	N	N
35	41	N	N	N	N	P
	42	N	N	N	N	N

SUBSTITUTE SHEET

-31-

		Antigens				
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
	43	N	N	N	N	N
	44	N	N	N	N	N
5	45	N	N	N	N	N
	46	N	N	N	N	N
	47	P	P	N	N	P
	48	N	N	N	N	N
	49	N	N	N	N	N
10	50	N	N	N	N	N
	51	N	P	P	N	P
	52	N	N	N	N	N
	53	N	P	P	N	P
	54	P	P	P	P	N
15	55	N	N	N	N	N
	56	N	N	N	N	N
	57	N	N	N	N	N
	58	N	N	N	N	N
	59	N	N	N	N	N
20	60	N	N	N	N	N
	61	N	N	N	N	N
	62	N	N	N	N	N
	63	N	N	N	N	N
	64	N	N	N	N	N
25	65	N	N	N	N	N
	66	N	N	N	N	N
	67	N	N	N	N	N
	68	N	N	N	N	N
	69	N	N	N	N	N
30	70	P	P	P	P	P
	71	N	N	N	N	N
	72	N	N	N	N	N
	73	P	P	P	P	N
	74	N	N	N	N	N
35	75	N	N	N	N	N
	76	N	N	N	N	P

-32-

		Antigens				
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	77	N	N	N	N	N
	78	N	N	N	N	N
	79	N	N	N	N	N
	80	N	N	N	N	N
	81	N	N	N	N	N
10	82	N	N	N	N	N
	83	P	P	N	N	N
	84	N	N	P	N	N
	85	N	N	N	N	N
	86	P	P	P	P	N
15	87	N	N	N	N	N
	88	N	N	N	N	N
	89	P	P	P	P	P
	90	P	P	P	P	N
	91	N	N	N	N	P
20	92	P	P	P	N	N
	93	N	N	N	N	N
	94	N	N	N	N	N
	95	N	N	N	N	N
	96	N	N	N	N	N
25	97	N	N	N	N	N
	98	N	P	P	P	P
	99	P	P	P	P	P
	100	N	N	N	N	N
	101	P	P	P	P	P
30	102	N	N	N	N	N
	103	N	N	N	N	N
	104		N	N	N	N
	105	P	P	P	P	N
	106	N	N	N	N	N
35	107	N	N	N	N	N
	108	N	N	N	N	N
	109	P	P	P	P	P
	110	P	P	P	N	P

SUBSTITUTE SHEET

-33-

		Antigens				
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
	111	P	P	P	N	P
	112	N	N	N	N	N
5	113	P	P	P	P	P
	114	N	N	N	N	N
	115	N	N	N	N	N
	116	P	P	P	P	P
	117	N	N	N	N	N
10	118	N	N	N	N	N
	119	N	N	N	N	N
	120	P	P	P	P	P
	121	N	N	N	N	N
	122	N	P	P	N	P
15	123	N	N	N	N	N
	124	N	N	N	N	N
	125	N	N	N	N	N
	126	P	N	N	N	N
	127	N	N	N	N	N
20	128	N	N	N	N	N
	129	N	N	N	N	N
	130	P	P	P	P	N
	131	N	N	N	N	P
	132	N	N	N	N	N
25	133	N	N	N	N	N
	134	N	N	N	N	N
	135	N	N	N	N	N
	136	N	N	N	N	N
	137	N	N	N	N	N
30	138	N	N	N	N	N
	139	N	N	N	N	N
	140	P	N	N	N	N
	141	P	N	P	P	P
	142	N	N	N	N	N
35	143	N	N	N	N	N
	144	N	N	N	N	N

SUBSTITUTE SHEET

-34-

Antigens						
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	145	N	N	N	N	N
	146	N	N	N	N	N
	147	N	N	N	N	N
	148	N	N	N	N	N
	149	N	N	N	N	N
10	150	N	N	N	N	N
	151	N	N	N	N	N
	152	N	N	N	N	N
	153	N	N	N	N	N
	154	P	P	P	P	P
15	155	N	N	N	N	N
	156	N	N	N	N	N
	157	N	N	N	N	N
	158	N	N	N	N	N
	159	N	N	N	N	N
20	160	N	N	N	N	N
	161	P	P	P	P	P
	162	N	N	N	N	N
	163	N	N	N	N	N
	164	P	P	P	N	P
25	165	N	N	N	N	N
	166	P	P	P	N	P
	167	N	N	N	N	N
	168	N	N	N	N	N
	169	N	N	N	N	N
30	170	N	N	N	N	N
	171	N	N	N	N	N
	172	N	N	N	N	N
	173	N	N	N	N	N
	174	N	N	N	N	N
35	175	N	N	N	N	N
	176	N	N	N	N	N
	177	N	N	N	N	P
	178	N	N	N	N	N

SUBSTITUTE SHEET

-35-

Antigens						
	<u>Donor</u>	<u>C100</u>	<u>C33c</u>	<u>C22</u>	<u>S2</u>	<u>NS5</u>
5	179	N	N	N	N	N
	180	N	N	N	N	N
	181	N	N	N̄	N	N
	182	N	N	N	N	N
	183	P	P	P	P	P
10	184	N	N	N	N	N
	185	N	N	N	N	N
	186	N	N	N	N	N
	187	N	N	N	N	N
	188	N	P	P	N	N
15	189	N	N	N	N	N
	190	N	N	N	N	N
	191	N	N	N	N	N
	192	N	N	N	N	N
	193	N	N	N	N	N
20	194	N	N	N	N	N
	195	N	N	N	N	N
	196	N	N	N	N	N
	197	N	N	N	N	P
	198	P	P	P	N	N
25	199	N	N	N	N	P
	200	P	P	P	P	N

The results on the paid donors generally confirms the results from the sera of infected individuals.

30 Example 7: ELISA Determinations of HCV Antibodies Using Combination of HCV Antigens

Plates coated with a combination of C22 and C33c antigens are prepared as follows. A solution containing coating buffer (50mM Na Borate, pH 9.0), 21 ml/plate, BSA (25 micrograms/ml), C22 and C33c (2.50 micrograms/ml each) is prepared just prior to addition to the Removeawell

-36-

Immulon I plates (Dynatech Corp.). After mixing for 5 minutes, 0.2ml/well of the solution is added to the plates, they are covered and incubated for 2 hours at 37°C, after which the solution is removed by aspiration.

- 5 The wells are washed once with 400 microliters wash buffer (100 mM sodium phosphate, pH 7.4, 140 mM sodium chloride, 0.1% (W/V) casein, 1% (W/V) Triton x-100, 0.01% (W/V) Thimerosal). After removal of the wash solution, 200 microliters/well of Postcoat solution (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, 0.1% (w/v) casein, 3% sucrose and 2 mM phenylmethylsulfonylfluoride (PMSF)) is added, the plates are loosely covered to prevent evaporation, and are allowed to stand at room temperature for 30 minutes. The wells are then aspirated to remove the solution, and lyophilized dry overnight, without shelf heating. The prepared plates may be stored at 2-8°C in sealed aluminum pouches with dessicant (3 g Sorb-it™ packs).

- In order to perform the ELISA determination, 20 microliters of serum sample or control sample is added to a well containing 200 microliters of sample diluent (100 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM EDTA, 0.1% (W/V) Casein, 0.01% (W/V) Thimerosal, 1% (W/V) Triton X-100, 100 micrograms/ml yeast extract). The plates are sealed, and are incubated at 37°C for two hours, after which the solution is removed by aspiration, and the wells are washed three times with 400 microliters of wash buffer (phosphate buffered saline (PBS) containing 0.05% Tween 20). The washed wells are treated with 200 microliters of mouse anti-human IgG-horse radish peroxidase (HRP) conjugate contained in a solution of Ortho conjugate diluent (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, 50% (V/V) fetal bovine serum, 1% (V/V) heat treated horse serum, 1 mM $K_3Fe(CN)_6$, 0.05% (W/V) Tween 20, 0.02% (W/V) Thimerosal). Treatment is for 1 hour at 37°C, the solution is removed by aspiration, and

SUBSTITUTE SHEET

-37-

the wells are washed three times with 400 ml wash buffer, which is also removed by aspiration. To determine the amount of bound enzyme conjugate, 200 microliters of substrate solution (10 mg O-phenylenediamine dihydrochloride per 5 ml of Developer solution) is added. Developer solution contains 50 mM sodium citrate adjusted to pH 5.1 with phosphoric acid, and 0.6 microliters/ml of 30% H_2O_2 . The plates containing the substrate solution are incubated in the dark for 30 minutes at room temperature, the reactions are stopped by the addition of 50 microliters/ml 4N sulfuric acid, and the ODs determined.

In a similar manner, ELISAs using fusion proteins of C22 and C33c, and C22, C33c, and S2 and combinations of C22 and C100, C22 and S2, C22 and an NS5 antigen, C22, C33c, and S2, and C22, C100, and S2 may be carried out.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in the fields of molecular biology, immunology, and related fields are intended to be within the scope of the following claims.

25

30

35

-38-

Claims

We claim:

- 5 1. A combination of synthetic hepatitis C viral
(HCV) antigens comprising:
 (a) a first HCV antigen from the C domain; and
 (b) at least one additional HCV antigen
selected from the group consisting of
10 (i) an HCV antigen from the NS3 domain;
 (ii) an HCV antigen from the NS4 domain;
 (iii) an HCV antigen from the S domain;
and
 (iv) an HCV antigen from the NS5 domain.
15
2. A combination of synthetic hepatitis C viral
(HCV) antigens comprising:
 (a) a first HCV antigen consisting essentially
of the C domain; and
20 (b) a second HCV antigen from the NS3 domain.
3. The combination of claim 2 wherein the first
HCV antigen is C22 and the second HCV antigen is C33c.
- 25 4. The combination of claim 2 including
 (c) a third HCV antigen from the S domain.
5. The combination of claim 3 including
 (c) HCV antigen S2.
30
6. A combination of synthetic HCV antigens
comprising:
 (a) a first HCV antigen consisting essentially
of the C domain; and
35 (b) a second HCV antigen from the NS4 domain.

-39-

7. The combination of claim 6 wherein the first HCV antigen is C22 and the second HCV antigen is C100.

8. The combination of claim 6 including
(c) a third HCV antigen from the S domain.

9. The combination of claim 7 including
(c) HCV antigen S2.

10. The combination of claim 1, 2, 3, 4, 5, 6, 7, 8 or 9 wherein the combination is in the form of a fusion polypeptide.

11. The combination of claim 1, 2, 3, 4, 5, 6, 7, 8 or 9 wherein the combination is in the form of said first HCV antigen and said additional antigens individually bound to a common solid matrix.

12. The combination of claim 11 wherein the solid matrix is the surface of a microtiter plate well, a bead or a dipstick.

13. The combination of claim 1, 2, 3, 4, 5, 6, 7, 8 or 9 wherein the combination is in the form of a mixture of said first HCV antigen and said additional HCV antigen(s).

14. A method for detecting antibodies to hepatitis C virus (HCV) in a mammalian body component suspected of containing said antibodies comprising contacting said body component with the combination of synthetic HCV antigens of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 under conditions that permit antibody-antigen reaction and detecting the presence of immune complexes of said antibodies and said antigens.

-40-

15. A method for detecting antibodies to hepatitis C virus (HCV) in a mammalian body component suspected of containing said antibodies comprising contacting said body component with a panel of synthetic HCV antigens comprising:
- 5 HCV antigens comprising:
- (a) a first HCV antigen from the C domain; and
 - (b) at least one additional HCV antigen selected from the group consisting of
- 10 (i) an HCV antigen from the NS3 domain;
 - (ii) an HCV antigen from the NS4 domain;
 - (iii) an HCV antigen from the S domain;
- and
- (iv) an HCV antigen from the NS5 domain
- 15 under conditions that permit antibody-antigen reaction and detecting the presence of immune complexes of said antibodies and said antigens.

16. A kit for carrying out an assay for detecting antibodies to hepatitis C antigen (HCV) in a mammalian
- 20 body component suspected of containing said antibodies comprising in packaged combination:
- (a) the combination of synthetic HCV antigens of claim 1;
 - (b) standard control reagents; and
 - 25 (c) instructions for carrying out the assay.

30

35

FIG. 1A

341 GCCAGCCCCCTGATGGGGCGA
CGGTCGGGGGACTACCCCGCT

319 CACTCCACCATGAATCACTCCCTGTGAGGAAC TACTGTCTTCACGCAGAAAGCGTCTAG
GTGAGGTGGTACTTAGTGAGGGGACACTCCTTGATGACAGAGTGCGTCTTTCGCAGATC

CCATGGCGTTAGTATGAGTGTCTGTCAGCCTCCAGGACCCCCCTCCCGGAGAGCCCAT
GGTACCCGCAATCATCTACAGCAGTCTGAGGTCTGGGGGGAGGCCCTCTCTCGGTAT

--199 GTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCCAGGACGACGGGTCTTCTTTGGA
CACCAGACGCCCTTGGCCACTCATGTGGCCCTTAACGGTCTCTGCTGGCCCGAAGAAACCT

--139 TCAACCCGCTCAATGCCCTGGAGATTTGGGGCTGCCCGCAAGACTGCTAGCCGAGTAGT
AGTTGGCGGAGTTACGGACCTCTAAACCCGCACGGGGCGTTCTGACGATCGGCCTCATCA

-- 79 GTTGGGTCGCGAAAGGCCTTGTGGTACTGCCCTGATAGGTGCTTGCAGTGCCCCGGGAG
CAACCCAGCGCTTTCGGAAACACCATGACGGACTATCCACGAACGCTCAGGGGCCCTC

-- 19 GTCTCGTAGACCGTGCACC
CAGAGCATCTGGCACGTGG

Thr
Arg

MetSerThrAsnProLysProGlnLysLysAsnLysArgAsnThrAsnArgArgProGln
1
ATGAGCAGCAATCCTAAACCTCAAAATAAAACAAACGTAACACCAACCGTCGCCACAG
TACTCGTGCTTAGGATTGGAGTTTTTTTTTTGTTTGCATTGTGTTGGCAGCGGGTGTCT

AspValLysPheProGlyGlyGlyGlnIleValGlyGlyValTyrLeuLeuProArgArg
61 GACGTC AAGTTC CCGGGTGGCGGT CAGATCGTTGGTGGAGTTTACTTGTGCCGCGCAGG
CTGCAGTTCAAGGGCCCAACCGCCAGTCTAGCAACCACTCAAATGAACAACGGCGCGTCC

2 / 26

121 GlyProArgLeuGlyValArgAlaThrArgLysThrSerGluArgSerGlnProArgGly
GGCCCTAGATTGGGTGTCGCGGACGAGAAAGACTTCCGAGCGGTCCGAACCTCGAGGT
CCGGGATCTAACCCACACGCGCGTGTCTTCTGTAAGGCTGCCAGCGTTGGAGCTCCA

181 ArgArgGlnProIleProLysAlaArgArgProGluGlyArgThrTrpAlaGlnProGly
AGACGTACGCTATCCCAAGGCTCGTGGCCCGAGGGCAGGACCTGGGCTCAGCCCCGGG
TCTGCAGTCGGATAGGGGTTCCGAGCAGCCGGGCTCCCGTCTTGACCCGAGTCGGGCCC

241 TyrProTrpProLeuTyrGlyAsnGluGlyCysGlyTrpAlaGlyTrpLeuLeuSerPro
TACCCTTGCCCCCTCTATGGCAATGAGGGTGGGGTGGGGATGGCTCCTGTCTCCCC
ATGGGAACCGGGGAGATACCGTTACTCCGACGCCCAACCCGCTACCGAGGACAGAGGG

301 ArgGlySerArgProSerTrpGlyProThrAspProArgArgArgSerArgAsnLeuGly
CGTGGCTCTCGGCCCTAGCTGGGGCCCCACAGACCCCGCGTAGGTCGCGCAATTGGGT
GCACCGAGAGCCGGATCGACCCCGGGGTGTCTGGGGGCCCATCCAGCGCGTTAAACCCA

361 LysValIleAspThrLeuThrCysGlyPheAlaAspLeuMetGlyTyrIleProLeuVal
AAGGTCATCGATACCCCTACGTGCGGCTTCGCCGACCTCATGGGTACATACCGCTCGTC
TTCCAGTAGCTATGGGAATGCACGCCGAAGCGGCTGGAGTACCCCATGTATGGCGAGCAG

421 GlyAlaProLeuGlyGlyAlaAlaArgAlaLeuAlaHisGlyValArgValLeuGluAsp
GGCGCCCTCTTGGAGGCGCTGCCAGGGCCCTGGCGCATGGCGTCCGGTTCTGGAAGAC
CCGCGGGGAGAACCTCCGCGACGCTCCCGGACCGCGTACCGCAGGCCCAAGACCTTCTG

FIG. 1B

3 / 26

FIG. 1C

Thr

481 GlyValAsnTyrAlaThrGlyAsnLeuProGlyCysSerPheSerIlePheLeuLeuAla
 GCGTGAACTATGCAACAGGAACCTTCCTGGTGTCTTCTCTATCTTCTCTGGCC
 CCGCACTTGATACGTTGTCCCTTGGAAGGACCAACGAGAAAGATAGAAAGAACCCG

 541 LeuLeuSerCysLeuThrValProAlaSerAlaTyrGlnValArgAsnSerThrGlyLeu
 CTGCTCTCTTGCTTGACTGTGCCCCGCTTCGGCTACCAAGTGCCTCACTCCACGGGCTT
 GACGAGAGAACGAACTGACACGGGGCGAAGCCGGATGTTCAACGGTTGAGGTGCCCCGAA

 601 TyrHisValThrAsnAspCysProAsnSerSerIleValTyrGluAlaAlaAspAlaIle
 TACCACGTCAACCAATGATTGCCCTAACTCGAGTATTGTGTACGAGCGGCCGATGCCATC
 ATGGTGCAGTGGTTACTAACGGGATTGAGCTCATACACATGCTCCGCCGCTACGGTAG

 661 LeuHisThrProGlyCysValProCysValArgGluGlyAsnAlaSerArgCysTrpVal
 CTGCACACTCCGGGTGCGTCCCTTGGTTCGTGAGGGCAACGCCCTCGAGGTGTGGGTG
 GACGTGTGAGGCCCCACGACGGGAACGCAAGCACTCCCGTTGCGGAGCTCCACAACCCAC

 721 AlaMetThrProThrValAlaThrArgAspGlyLysLeuProAlaThrGlnLeuArgArg
 GCGATGACCCCTACGGTGGCCACCAAGGATGGCAAACTCCCCCGCAGCAGCTTCGACGT
 CGCTACTGGGGATGCCACCGGTGGTCCCTACCGTTTGAGGGCGCTGCGTGAAGCTGCA

 781 HisIleAspLeuLeuValGlySerAlaThrLeuCysSerAlaLeuTyrValGlyAspLeu
 CACATCGATCTGCTTGTTCGGAGCGCCACCCCTCTGTTCGGCCCTCTACGTGGGGACCTA
 GTGTAGCTAGACGAACAGCCCTCGCGGTGGGAGACAAAGCCGGGAGATGCACCCCTGGAT

 841 CysGlySerValPheLeuValGlyGlnLeuPheThrPheSerProArgArgHisTrpThr
 TCGGGGTCTGTCTTCTTGTTCGGCCAACTGTTCACCTTCTCTCCAGCGCCACTGGACG
 ACGCCACAGACAGAAAGAACGCCGTTGACAAGTGAAGAGAGGGTCCGCGGTGACCTGC

4 / 26

901 ThrGlnGlyCysAsnCysSerIleTyrProGlyHisIleThrGlyHisArgMetAlaTrp
ACGCAAGGTTGCAATTGCTCTATCTATCCCGCCATATAACGGTCACCGCATGGCATGG
TGCCTTCCAAACGTTAACGAGATAGATAGGGCCGGTATATTGCCCAGTGCGGTACCGTACC

961 AspMetMetMetAsnTrpSerProThrAlaLeuValMetAlaGlnLeuLeuArgIle
Val
GATATGATGATGAACCTGGTCCCTACGACGGCGTTGGTAATGGCTCAGCTGCTCCGGATC
CTATACTACTTGAACCGGGGATGCTGCCGAACCATTAACGAGTCGACGAGGCCTAG

1021 ProGlnAlaIleLeuAspMetIleAlaGlyAlaHisTrpGlyValLeuAlaGlyIleAla
CCACAAGCCATCTTGGACATGATCGCTGGTCTCACTGGGGAGTCTCTGGCGGCATAGCG
GGTGTTCCGGTAGAACCTGTACTAGCGACCAACGAGTGACCCCTCAGGACCGCCCGTATCGC

1081 TyrPheSerMetValGlyAsnTrpAlaLysValLeuValValLeuLeuPheAlaGly
TATTTCTCCATGGTGGGAACTGGCGGAAGTCTCTGGTAGTCTGCTGCTATTGCCCGGC
ATAAAGAGGTACCAACCCCTTGACCCGCTTCCAGGACCATCACGACGATATAACGGCCG

1141 ValAspAlaGluThrHisValThrGlyGlySerAlaGlyHisThrValSerGlyPheVal
GTCGACGCGGAAACCCACGTCACCGGGGAAGTGCCGGCCACACTGTGTCTGGATTGTT
CAGCTGCGCCCTTTGGGTGCAGTGGCCCCCTTCACGGCCGGTGTGACACAGACCTAAACAA

1201 SerLeuLeuAlaProGlyAlaLysGlnAsnValGlnLeuIleAsnThrAsnGlySerTrp
AGCCTCCTCGCACCGAGCGCCAAAGCAGAACGTCCAGCTGATCAACACCAACGCGAGTTGG
TCGGAGGAGCGTGTCCCGGGTTCGTCTTGCAGGTCCGACTAGTTGTGTTGCCGTCAACC

FIG. 1D

5 / 26

FIG. 1E

1261 HisLeuAsnSerThrAlaLeuAsnCysAsnAspSerLeuAsnThrGlyTrpLeuAlaGly
 CACCTCAATAGCACGGCCCTGAACCTGCAATGATAGCTCAACACCGGCTGGTTGGCAGGG
 GTGGAGTTATCGTGCCGGGACTTGACGTTACTATCGGAGTTGTGGCCGACCAACCGTCCC

 1321 LeuPheTyrHisLysPheAsnSerSerGlyCysProGluArgLeuAlaSerCysArg
 CTTTTCATATCACCAAGTTCAACTCTTCAGGCTGTCTGAGAGGCTAGCCAGCTGCCGA
 GAAAGATAGTGGTGTTCAGTTGAGAAAGTCCGACAGGACTCTCCGATCGGTCTGACGGCT

 1381 ProLeuThrAspPheAspGlnGlyTrpGlyProIleSerTyrAlaAsnGlySerGlyPro
 CCCCTTACCGATTGACCCAGGCTGGGGCCCTATCAGTTATGCCAACGGAAGCGGCCCC
 GGGAAATGGCTAAAACTGGTCCCGACCCCGGATAGTCAATACGGTTGCCCTTCGCCGGGG

 1441 AspGlnArgProTyrCysTrpHisTyrProProLysProCysGlyIleValProAlaLys
 GACCAAGCCCCCTACTGTCTGGCACTACCCCCCAAACCTTGCGGTATTGTGCCCGGAAG
 CTGGTCGCGGGGATGACGACCGTGATGGGGGTTTGGAAACGCCATAACACGGGGCGCTTC

 1501 SerValCysGlyProValTyrCysPheThrProSerProValValGlyThrThrAsp
 AGTGTGTGTGTCGGTATATTGCTTCACTCCACGCCCGTGGTGGGAAACGACCCGAC
 TCACACACACAGGCCATATAACGAAGTGAGGGTCGGGGCACCAACCTTGCTGGCTG

 1561 ArgSerGlyAlaProThrTyrSerTrpGlyGluAsnAspThrAspValPheValLeuAsn
 AGGTCGGGCGCGCCACCTACAGCTGGGGTGAAATGATACGGACGCTCTTCGTCCTTAAC
 TCCAGCCCGCGGGTGATGTCTGACCCCACTTTTACTATGCTGCTGCAGAACGGAATTG

 1621 AsnThrArgProProLeuGlyAsnTrpPheGlyCysThrTrpMetAsnSerThrGlyPhe
 AATACCAAGGCCACCGCTGGGCAATTGGTTCGGTTGTACCTGGATGAACCTCAACTGGATTC
 TTATGGTCCGGTGGCGACCCGTTAACCAAGCCAACATGACCTACTTGAGTTGACCTAAG

6 / 26

1681 ThrLysValCysGlyAlaProProCysValIleGlyGlyAlaGlyAsnAsnThrLeuHis
 ACCAAAGTGTGCGAGCGCTCTTGTGTCTATCGAGGGCGGCAACAAACCCCTGCCAC
 TGGTTTCACACGCGCTCGCGAGGAACACAGTAGCCTCCCCCGCTTGTGTGGGACGTG

 1741 CysProThrAspCysPheArgLysHisProAspAlaThrTyrSerArgCysGlySerGly
 TGCCCCACTGATTGCTTCCGCAAGCATCCGGACGCCACATACTCTCGGTGGCTCCGGT
 ACGGGTGACTAACGAAGCGTTCGTAGGCGCTGCGGTGTATGAGAGCCACGCCGAGGCCA

 Ile
 1801 ProTrpLeuThrProArgCysLeuValAspTyrProTyrArgLeuTrpHisTyrProCys
 CCCTGGATCACACCCAGGTGCTGGTCGACTACCCGTATAGGCTTTGGCATTATCCTTGT
 GGGACCTAGTGTGGTCCACGGACCGAGCTGTATCCGAAACCGTAATAGGAACA

 1861 ThrIleAsnTyrThrIlePheLysIleArgMetTyrValGlyGlyValGluHisArgLeu
 ACCATCAACTACACCATATTTAAATCAGGATGTACGTGGGAGGGTCCGAACACAGGCTG
 TGGTAGTTGATGTGGTATAAATTTTAGTCTACATGCACCCCTCCCCAGCTTGTGTCCGAC

 1921 GluAlaAlaCysAsnTrpThrArgGlyGluArgCysAspLeuGluAspArgAspArgSer
 GAAGCTGCCCTGCAACTGGACCGCGGGCGGAACGTTGCCATCTGGAAGACAGGGACAGGTCC
 CTTCGACGGACGTTGACCTGCGGCGCCGCTTGCAACGCTAGACCTTCTGTCTCCCTGCCAGG

 1981 GluLeuSerProLeuLeuThrThrThrGlnTrpGlnValLeuProCysSerPheThr
 GAGCTCAGCCCGTTACTGCTGACCACTACACAGTGGCAGGTCTCCGTGTTCTCTCACA
 CTCGAGTCGGGCAATGACGACTGGTGATGTGTACCGTCCAGGAGGGCACAAAGGAAGTGT

FIG. 1F

7/26

FIG. 1G

2041 ThrLeuProAlaLeuSerThrGlyLeuIleHisLeuHisGlnAsnIleValAspValGln
 ACCCTACCAGCCTTGTCACCGCCTCATCCACCTCCACCAGAACATTTGTGGACGTGCAG
 TGGGATGGTCGGAACAGGTGGCCGGAGTAGGTGGAGGTGCTTGTAAACACCTGCACGTC

 2101 TyrLeuTyrGlyValGlySerSerIleAlaSerTrpAlaIleLysTrpGluTyrValVal
 TACTTGTAACGGGTGGGTCAAGCATCGCTCCTGGGCCATTAAAGTGGAGTACGTCTGTT
 ATGAACATGCCCCACCCAGTTCTGTAGCGCAGGACCCGGTAATTCAACCTCATGCAGCAA

 2161 LeuLeuPheLeuLeuAlaAspAlaArgValCysSerCysLeuTrpMetMetLeuLeu
 CTCCTGTTCCTTCTGCTTGACAGACCGCGCGTCTGCTCCTGCTTGTGGATGATGCTACTC
 GAGGACAAAGGAAGACGAACGTCTGCGCGCGCAGACGAGGACGAACACCTACTACGATGAG

 2221 IleSerGlnAlaGluAlaAlaLeuGluAsnLeuValIleLeuAsnAlaAlaSerLeuAla
 ATATCCCAAGCGGAGCGGCTTTGGAGAACCTCGTAATACTAATGCAGCATCCCTGGCC
 TATAGGGTTCCGCTCCGCCGAAACCTCTTGGAGCATTTATGAATTACGTCGTAGGGACCGG

 2281 GlyThrHisGlyLeuValSerPheLeuValPhePheCysPheAlaTrpTyrLeuLysGly
 GGGACGCACCGTCTTGATATCCTTCTCCTCGTGTCTTCTGCTTGCATGGTATTGAAGGT
 CCTGCGTGCCAGAACATAGGAAGGAGCACAAAGAACGAAACGTACCATAAACTTCCCA

 2341 LysTrpValProGlyAlaValTyrThrPheTyrGlyMetTrpProLeuLeuLeuLeu
 AAGTGGGTGCCCCGAGCGGTCTACACCTTCTACGGGATGTGGCCTCTCCTCCTGCTCCTG
 TTACCCACGGGCTCGCCAGATGTGGAAGATGCCCTACACCGGAGAGGACGAGGAC

 2401 LeuAlaLeuProGlnArgAlaTyrAlaLeuAspThrGluValAlaAlaSerCysGlyGly
 TTGGCGTTGCCCCAGCGGGGTACCGCTGGACACGGAGGTGGCCCGCTCGTGTGGCGGT
 AACCGCAACGGGTGCGCCCGCATGCGCGACCTGTGCTCCACCGGCGCAGCACACCGCCA

8 / 26

2461 ValValLeuValGlyLeuMetAlaLeuThrLeuSerProTyrTyrLysArgTyrIleSer
 GTTGTTCCTCGTCGGTTGATGGCGTGA CTCTGTACCATATTACAAGCGCTATATCAGC
 CAACAAGAGCAGCCCAACTACCGCGACTGAGACAGTGGTATAATGTTCCGGATATAGTCG

 2521 TrpCysLeuTrpTrpLeuGlnTyrPheLeuThrArgValGluAlaGlnLeuHisValTrp
 TGGTGCTTGTGTGGCTTCAGTATTTTCTGACCAGAGTGGAAGCGCAACTGCACGTGTGG
 ACCACGAACACCAACCGAAGTCATAAAAGACTGGTCTCACCTTCGCCGTTGACGTGCACACC
 (Asn)
 IleProProLeuAsnValArgGlyGlyArgAspAlaValIleLeuLeuMetCysAlaVal
 2581 ATTCCCCCCTCAACGTCGAGGGGGCGGCGACGCCGTCACTTACTCATGTGTGCTGTA
 TAAGGGGGGAGTTGCAGGCTCCCCCGCGCTGGCGCAGTAGAATGAGTACACACGACAT
 HisProThrLeuValPheAspIleThrLysLeuLeuAlaValPheGlyProLeuTrp
 2641 CACCCGACTCTGGTATTGACATCACCAAAATTGCTGCTGGCCGCTTCGGACCCCTTTGG
 GTGGGCTGAGACCATAAACTGTAGTGGTTTAAACGACGACCGGCAGAACCTGGGGAAACC
 IleLeuGlnAlaSerLeuLeuLysValProTyrPheValArgValGlnGlyLeuLeuArg
 2701 ATTCTTCAAGCCAGTTTGCTTAAAGTACCCCTACTTGTGCGCGTCCAAAGGCCTTCTCCGG
 TAAGAAAGTTCGGTCAACCGAATTTCATGGGATGAACAACGCGCAGGTTCCGGAGAGGCC
 PheCysAlaLeuAlaArgLysMetIleGlyGlyHisTyrValGlnMetValIleIleLys
 2761 TTCTGCGCGTTAGCGGGAAGATGATCGGAGGCCATTACGTGCAAAATGGTCATCATTAAG
 AAGACGCGCAATCGCGCCTTCTACTAGCCTCCGGTAATGCACGTTTACCAGTAGTAATTC

FIG. 1H

9 / 26

FIG. 11

2821 LeuGlyAlaLeuThrGlyThrTyrValTyrAsnHisLeuThrProLeuArgAspTrpAla
TTAGGGCGCTTACTGGCACCTATGTTTATAACCATCTCACTCCTCTTCGGGACTGGGCG
AATCCCCGCGAATGACCGTGGATACAAATATTGGTAGAGTGAGGAGAGCCCTGACCCGC
HisAsnGlyLeuArgAspLeuAlaValAlaValGluProValValPheSerGlnMetGlu
2881 CACAACGGCTTGCAGATCTGGCGTGGCTGTAGAGCCAGTCGTCTCTCCAAATGGAG
GTGTTGCCGAACGCTCTAGACCGGCACCGACATCTCGGTACGACAGAGGGTTACCTC
ThrLysLeuIleThrTrpGlyAlaAspThrAlaAlaCysGlyAspIleIleAsnGlyLeu
2941 ACCAAGCTCATCACGTGGGGGAGATACCGCCGCGTGGGTGACATCATCAACGGCTTG
TGGTTCGAGTAGTGCAACCCCCGCTCTATGGCGGCGCACGCCACTGTAGTAGTTGCCGAAC
ProValSerAlaArgArgGlyArgGluIleLeuLeuGlyProAlaaspGlyMetValSer
3001 CCTGTTCCGCCCGCAGGGCCGGAGATACTGCTCGGGCCAGCCGATGGAATGGTCTCC
GGACAAAGCGGGCGTCCCCGCCCTCTATGACGAGCCCCGGTCCGTACCTTACCAGAGG
LysGlyTrpArgLeuLeuAlaProIleThrAlaTyrAlaGlnGlnThrArgGlyLeuLeu
3061 AAGGGTGGAGGTGCTGGCGCCCATCACGGCGTAGCGCCAGCAGACAAAGGGCCCTCCTA
TTCCCCACCTCCAAACGACCGCGGTAGTGCCGCGCATGCGGGTCTGTGTCCCCGGAGGAT
GlyCysIleIleThrSerLeuThrGlyArgAspLysAsnGlnValGluGlyGluValGln
3121 GGGTGCATAATCACCCAGCCCTAACTGGCCGGACAAAACCAAGTGAGGGTGAGGTCCAG
CCCACGTATTAGTGGTGGATTGACCGGCCCTGTTTTGTGTTCACTCCCACTCCAGGTC
IleValSerThrAlaAlaGlnThrPheLeuAlaThrCysIleAsnGlyValCysTrpThr
3181 ATTGTGTCAACTGCTGCCCAACCTTCCTGGCAACGTGCATCAATGGGTGTGCTGGACT
TAACACAGTTGACGACGGGTTTGGGAAGGACCGTTGCACGTAGTTACCCCAACACGACCTGA

10 / 26

3241 ValTyrHisGlyAlaGlyThrArgThrIleAlaSerProLysGlyProValIleGlnMet
GTCTACCAAGGGCCGGAACGAGGACCATCGCGTCACCCAAAGGTCTCTGTATCCAGATG
CAGATGGTGCCCGGCTTGTCTCTGGTAGCGCAGTGGTTCCAGGACAGTAGGTCTAC

3301 TyrThrAsnValaspGlnaspLeuValGlyTrpProAlaProGlnGlySerArgSerLeu
TATACCAATGTAGACCAAGACCTTGTGGGTGGCCGCTCCGCAAGTAGCCGCTCATTTG
ATATGGTTACATCTGTGTTCTGGAACACCCGACCCGCGGAGCGTTCCATCGCGGAGTAAC

3361 ThrProCysThrCysGlySerSerAspLeuTyrLeuValThrArgHisAlaAspValIle
ACACCCCTGCACTTGGGCTCCTCGGACCTTTACCTGGTCAAGGACGACGCGGATGTCAAT
TGTGGGACGTGAACGCCGAGGAGCCTGGAAATGGACCAAGTGTCCGTGCGGCTACAGTAA

3421 ProValArgArgGlyAspSerArgGlySerLeuLeuSerProArgProIleSerTyr
CCCGTGCGCGCGGGGTGATAGCAGGGGCGAGCCTGTGTGCGCCCGGCCCATTTCTCTAC
GGGCACGGCGCGCGCCCACTATCGTCCCCGTCGGACGACAGCGGGGCGGGTAAAGGATG

3481 LeuLysGlySerSerGlyGlyProLeuLeuCysProAlaGlyHisAlaValGlyIlePhe
TTGAAAGGCTCTCGGGGGTCCGCTGTTGTGCCCCCGGGGCGACGCGGTGGCATATTT
AACTTCCGAGAGCCCCCGAGGCAACACGCGGGCGCCCCGTGCGGCAACCCGTATAAA

3541 ArgAlaAlaValCysThrArgGlyValAlaLysAlaValAspPheIleProValGluAsn
AGGGCCCGGTGTGCACCCGTGGAGTGCTAAGCGGTGGACTTTATCCCTGTGGAGAAC
TCCCGGCGCCACACGTGGGCACTCACCGATTCCGCCACCTGAAATAGGGACACCTCTTG

FIG. 1J

11 / 26

FIG. 1K

3601 LeuGluThrThrMetArgSerProValPheThrAspAsnSerSerProProValValPro
 CTAGAGACAACCATGAGGTCCCGGTGTTCACGGATAACTCTCTCCACCAAGTAGTGCCC
 GATCTCTGTGTACTCCAGGGGCCACAAGTGCCTATTGAGGAGAGGTGTCATCACGGG

 3661 GlnSerPheGlnValAlaHisLeuHisAlaProThrGlySerGlyLysSerThrLysVal
 CAGAGCTTCCAGGTGGCTCACCTCCATGCTCCACAGGCAGCGCAAAAGCACCAAGGTC
 GTCTCGAAGGTCCACCGAGTGGAGGTACGAGGGTGTCCGTCCCGCTTTCGTGTTCCAG

 3721 ProAlaAlaTyrAlaAlaGlnGlyTyrLysValLeuValLeuAsnProSerValAlaAla
 CCGGCTGCATATGCAGCTCAGGGCTATAAGGTGCTAGTACTCAACCCCTCTGTGCTGCA
 GGCCGACGTATACGTCGAGTCCCGATATATCCACGATCATGAGTTGGGAGACAAACGCGT

 3781 ThrLeuGlyPheGlyAlaTyrMetSerLysAlaHisGlyIleAspProAsnIleArgThr
 ACAC TGGGCTTGGTGCTTACATGTCCAAGGCTCATGGGATCGATCCTAACATCAGGACC
 TGTGACCCGAAACCAACGAATGTACAGGTTCCGAGTACCCTAGCTAGGATTGTAGTCCCTGG

 3841 GlyValArgThrIleThrThrGlySerProIleThrTyrSerThrTyrGlyLysPheLeu
 GGGGTGAGAACAAATTACCACTGGCAGCCCCCATCACGTACTCCACCTACGGCAAGTTCCTT
 CCCCACCTCTTGTTAATGGTGACCGTCCGGGTAGTGTCATGAGGTGGATGCCGTTCAAGGAA

 3901 AlaAspGlyGlyCysSerGlyAlaTyrAspIleIleIleCysAspGluCysHisSer
 GCCGACGGCGGTGCTCGGGGGCGCTTATGACATAATAATTGTGACGAGTGCCACTCC
 CGGCTGCCGCCACGAGCCCCCGCGAATACTGTATTATTAAACACTGCTCACGGTGAGG

12 / 26

3961 ThrAspAlaThrSerIleLeuGlyIleGlyThrValLeuAspGlnAlaGluThrAlaGly
 (Val)
 ACGGATGCCACATCCATCTTGGGCATCGGCATGTCTTGACCAAGCAGAGACTGCGGGG
 TGCCTACGGTGTAGGTAGAACCCGTAGCCGTGACAGGAACTGGTTCGTCTCTGACGCCCC
 4021 AlaArgLeuValValLeuAlaThrAlaThrProProGlySerValThrValProHisPro
 GCGAGACTGGTGTGTCTCGCCACCGCCACCCCTCCGGGCTCCGTCACGTGCCCCATCCC
 CGCTCTGACCAACACGAGCGGTGGGTGGGAGGCCCGAGGCAGTGACACGCGGTAGGG
 4081 AsnIleGluGluValAlaLeuSerThrThrGlyGluIleProPheTyrGlyLysAlaIle
 AACATCGAGGAGGTGTCTCTGTCCACCCGAGAGATCCCTTTTACGGCAAGGCTATC
 TTGTAGCTCCTCCAACGAGACAGGTGGTGGCTCTCTAGGGAAAAATGCCGTTCGGATAG
 4141 ProLeuGluValIleLysGlyGlyArgHisLeuIlePheCysHisSerLysLysCys
 CCCCTCGAAGTAATCAAGGGGGGAGACATCTCATCTTCTGTCAATCAAGAAAGAGTGC
 GGGAGCTTCATTAGTTCCCCCTCTGTAGAGTAGAAGACAGTAAGTTTCTTCTTCACG
 4201 AspGluLeuAlaAlaLysLeuValAlaLeuGlyIleAsnAlaValAlaTyrTyrArgGly
 GACGAACCTCGCCGCAAGCTGTGTCGCAATGGGCATCAATGCCGTGGCCTACTACCGCGGT
 CTGCTTGAGCGCGCTTTCGACCAGCGTAACCCGTAGTTACGGCACCGGATGATGGCGCCA
 4261 LeuAspValSerValIleProThrSerGlyAspValValValAlaThrAspAlaLeu
 CTTGACGTGTCCGTATCCCGACCGAGCGGCGATGTGTGTCGTGGCAACCGATGCCCTC
 GAACTGCACAGGCAGTAGGGCTGGTCCCGCTACACAGCAGCACCGTTGGCTACGGGAG
 Tyr
 4321 MetThrGlyTyrThrGlyAspPheAspSerValIleAspCysAsnThrCysValThrGln
 ATGACCCGGCTATACCGGCACTTCGACTCGGTGATAGACTGCAATACGTGTGTACCCAG
 TACTGGCCGATATGGCCGCTGAAGCTGAGCCACTATCTGACGTTATGCCACACAGTGGGTC

FIG. 1L

13 / 26

FIG. 1M

(Ser)

ThrValAspPheSerLeuAspProThrPheThrIleGluThrIleThrLeuProGlnAsp
ACAGTCGATTTCAGCCTTGACCCCTACCTTCACCATTGAGACAATCACGCTCCCCCAGGAT
TGTCAGCTAAAGTCGGAACCTGGGATGGAAGTGTAACCTCTGTAGTGCAGGGGGTCTTA

4381

AlaValSerArgThrGlnArgGlyArgThrGlyArgGlyLysProGlyIleTyrArg
GCTGTCTCCCGCACTCAACGTCGGGGCAGGACTGGCAGGGGGAAGCCAGGCATCTACAGA
CGACAGAGGGCGTGAGTTGCCAGCCCCGCTCCTGACCGTCCCCCTTCGGTCCGTAGATGTCT

4441

PheValAlaProGlyGluArgProSerGlyMetPheAspSerSerValLeuCysGluCys
TTTGTGGCACCCGGGGAGCGCCCCCTCCGGCATGTTCCGACTCGTCCGCTCTGTGTAGTGC
AAACACCGTGGCCCCCTCGCGGGGAGGCCGTACAAAGCTGAGCAGGAGGACACTCACG

4501

TyrAspAlaGlyCysAlaTrpTyrGluLeuThrProAlaGluThrThrValArgLeuArg
TATGACGCAGGCTGTGCTTGGTATGAGCTCACGCCCGCCGAGACTACAGTTAGGCTACGA
ATACTGCGTCCGACACGAAACCATACTCGAGTGGGGCGGCTCTGTATGTCAATCCGATGCT

4561

AlaTyrMetAsnThrProGlyLeuProValCysGlnAspHisLeuGluPheTrpGluGly
GCGTACATGAACACCCCGGGGCTTCCCGTGTGCCAGGACCATCTTGAATTTGGGAGGGC
CGCATGTACTTGTGGGGCCCCGGAAGGGCACACGGTCTCTGTAGAACTTAAACCCCTCCCCG

4621

ValPheThrGlyLeuThrHisIleAspAlaHisPheLeuSerGlnThrLysGlnSerGly
GTCTTTACAGGCCTCACTCATATAGATGCCCACTTTCTATCCCAGACAAAGCAGAGTGGG
CAGAAATGTCCGGAGTGAGTATATCTACGGGTGAAGATAGGGTCTGTTCGTCTCACCC

4681

GluAsnLeuProTyrLeuValAlaTyrGlnAlaThrValCysAlaArgAlaGlnAlaPro
GAGAACCTTCCCTTACCTGGTAGCGTACCAAGCCACCGTGTGCGCTAGGGCTCAAGCCCCCT
CTCTTGGGAAGGAATGGACCATCGCATGTGTTCCGTGGCACACGCGATCCCCGAGTTCGGGGA

4741

14 / 26

4801 ProProSerTrpAspGlnMetTrpLysCysLeuIleArgLeuLysProThrLeuHisGly
CCCCCATCGTGGACACAGATGTGGAAGTGTATTGATTCGCTCAAGCCACCCCTCCATGGG
GGGGTAGCACCCCTGGTCTACACCTTCACAAACTAAGCGGAGTTCGGGTGGAGGTACCC

4861 ProThrProLeuTyrArgLeuGlyAlaValGlnAsnGluIleThrLeuThrHisPro
CCAACACCCCTGCTATACAGACTGGCGCTGTTTTCAGAAATGAAATCACCTGACGCACCCA
GGTTGTGGGACGATATGTCTGACCCCGACAAAGTCTTACTTTAGTGGGACTGCCGTGGGT

4921 ValThrLysTyrIleMetThrCysMetSerAlaAspLeuGluValValThrSerThrTrp
GTCACCAAAATACATCATGACATGTCATGTCGCCGACCTGGAGTTCGTACGAGCACCTGG
CAGTGGTTTATGTAGTACTGTACGTACAGCCGGCTGGACCTCCAGCAGTGTCTCGTGGACC

4981 ValLeuValGlyGlyValLeuAlaAlaLeuAlaAlaTyrCysLeuSerThrGlyCysVal
GTGCTCGTTGGCGGCTCCTGGCTGCTTGGCCGCGTATTGCCGTCAACAGGCTGCCGTG
CACGAGCAACCGCCGACGACCGACGAAACCGGCGCATAACGGACAGTTGTCCGACGCAC

5041 ValIleValGlyArgValValLeuSerGlyLysProAlaIleIleProAspArgGluVal
GTCATAGTGGCAGGTCGTCTTGTCCGGGAAGCCGGCAATCATACCTGACAGGGAAGTC
CAGTATCACCCGTCCAGCAGAAACAGGCCCTTCGGCCGTTAGTATGGACTGTCCCTTCAG

5101 LeuTyrArgGluPheAspGluMetGluGluCysSerGlnHisLeuProTyrIleGluGln
CTCTACCGAGAGTTCGATGAGATGGAAGAGTGTCTCTCAGCACTTACCGTACATCGAGCAA
GAGATGGCTCTCAAGCTACTCTACCTTCTCAGCAGAGTCTGTAATGGCATGTAGTCTGTT

FIG. 1N

15/ 26

FIG. 10

5161 GlyMetMetLeuAlaGluGlnPheLysGlnLysAlaLeuGlyLeuLeuGlnThrAlaSer
 GGGATGATGCTCGCCGAGCAGTTCAAGCAGAGGCCCTCGGCTCTCTGCAGACCGCGTCC
 CCCTACTACGAGCGGCTCGTCAAGTTCGTCTTCCGGGAGCCGAGGACGTCTGGCGCAGG

 5221 ArgGlnAlaGluValIleAlaProAlaValGlnThrAsnTrpGlnLysLeuGluThrPhe
 CGTCAGGCAGAGGTTATCGCCCTGCTGTCCAGACCAACTGGCAAAACTCGAGACCTTC
 GCAGTCCGCTCTCCAATAGCGGGGACGACAGGTCTGGTTGACCGTTTGTGAGCTCTGGAAG

 5281 TrpAlaLysHisMetTrpAsnPheIleSerGlyIleGlnTyrLeuAlaGlyLeuSerThr
 TGGGCCAAGCATATGTGGAACCTTCATCAGTGGGATACAATACTTGGCGGCTTGTC AACG
 ACCCGCTTCGTATACACCTTGAAGTAGTCAACCTATGTATGAACCCGCCGAACAGTTGC

 5341 LeuProGlyAsnProAlaIleAlaSerLeuMetAlaPheThrAlaAlaValThrSerPro
 CTGCCCTGGTAACCCCGCATTTGCTTCATTTGATGGCTTTTACAGCTGCTGTACACGCCCCA
 GACGGACCATTTGGGGCGGTAAACGAAGTAACTACCGAAATGTCTGACGACAGTGGTCCGGT

 5401 LeuThrThrSerGlnThrLeuLeuPheAsnIleLeuGlyGlyTrpValAlaAlaGlnLeu
 CTAACCACTAGCCAAACCTCCTCTTCAACATATTTGGGGGGTGGTGGCTGCCACGCTC
 GATTGGTGATCGGTTTGGGAGGAGAAAGTTGTATAACCCCCCACCCACCGACGGGTCCGAG

 5461 AlaAlaProGlyAlaAlaThrAlaPheValGlyAlaGlyLeuAlaGlyAlaAlaIleGly
 GCCGCCCCCGGTGCCGCTACTGCCCTTGTGGCGCTGGCTTAGCTGGCGCCCATCGGC
 CGCGGGGGCCACGGCGATGACGGAAACACCCCGGACCGAATCGACCCGCGGTAGCCG

 5521 SerValGlyLeuGlyLysValLeuIleAspIleLeuAlaGlyTyrGlyAlaGlyValAla
 AGTGTGGACTGGGGAAGTCTCTCATAGACATCCTTGCAGGTATGGCGCGGTGGCGG
 TCACAACTGACCCCTTCCAGGAGTATCTGTAGGAACGTCCCATACCGCGCCCGCACCCG

16 / 26

5581 GlyAlaLeuValAlaPheLysIleMetSerGlyGluValProSerThrGluAspLeuVal
(Gly)
GGAGCTCTTGTTGGCATTCAAGATCATGAGCGGTGAGTCCCTCCACGGAGACCTGGTC
CCTCGAGAACACCGTAAGTTCTAGTACTCGCCACTCCAGGGGAGGTGCTCTCTGGACCAG

5641 AsnLeuLeuProAlaIleLeuSerProGlyAlaLeuValValGlyValValCysAlaAla
AATCTACTGCCCGCATCTCTCGCCGGAGCCCTCGTAGTCGGCGTGGTCTGTGCAGCA
TTAGATGACGGGGGTAGGAGAGCGGGCCTCGGGAGCATCAGCCGCACACGACACGTCGT

5701 IleLeuArgArgHisValGlyProGlyGluGlyAlaValGlnTrpMetAsnArgLeuIle
ATACTGCGCCGGCACGTTGGCCCGGGAGGGGAGTGCACTGATGAACCGGCTGATA
TATGACGGGGCGGTGCAACCGGGCCCGTCCCCGTCACGTCACTACTTGGCCGACTAT

5761 AlaPheAlaSerArgGlyAsnHisValSerProThrHisTyrValProGluSerAspAla
GCCTTCGCCCTCCCGGGGAACCATGTTTCCCCCACGCACTACGTGCCGGAGAGCGATGCA
CGGAAGCGGAGGGCCCCCTTGGTACAAAGGGGTGCGTGATGCACGGCCCTCTCGCTACGT

5821 (HisCys)
AlaAlaArgValThrAlaIleLeuSerSerLeuThrValThrGlnLeuLeuArgArgLeu
GTCGCCCGCGTCACTGCCATACTCAGCAGCCCTCACTGTAAACCCAGCTCCTGAGCGCACTG
CGACGGCGCAGTGACGGTATGAGTCGTGCGAGTGACATTGGGTGCGAGACTCCGCTGAC

5881 HisGlnTrpIleSerSerGluCysThrThrProCysSerGlySerTrpLeuArgAspIle
CACCAGTGGATAAGCTCGGAGTGATCACTCCATGCTCCGGTTCCTGGCTAAGGGACATC
GTGGTCACCTATTTCGAGCCTCACATGGTGAGGTACGAGGCCAAGGACCGATTCCCTGTAG

FIG. 1P

17 / 26

FIG. 1Q

5941 TrpAspTrpIleCysGluValLeuSerAspPheLysThrTrpLeuLysAlaLysLeuMet
 TGGCACTGATATCGAGGTGTGAGCGACTTAAGACCTGGCTAAAGCTAAGCTCATG
 ACCCTGACCTATACGCTCCACAACCTCGCTGAAATTCTGGACCGGATTTTCGATTCCGAGTAC

 6001 ProGlnLeuProGlyIleProPheValSerCysGlnArgGlyTyrLysGlyValTrpArg
 CCACAGCTGCCTGGATCCCTTTGTGTCTCTGCCAGCGGGTATAAGGGGTCTGGCGGA
 GGTGTCGACGGACCCCTAGGGGAAACACAGGACGGTCCGCCCATATTCCCCAGACCGCT

 6061 (Val)
 GlyAspGlyIleMethIleThrArgCysHisCysGlyAlaGluIleThrGlyHisValLys
 GTGGACGGCATCATGCACACTCGCTGCCACTGTGGAGCTGAGATCACTGGACATGTCAAA
 CACCTGCCGTAGTACGTGTGAGCGACGGTGACACCTCGACTCTAGTGACCTGTACAGTTT

 6121 AsnGlyThrMetArgIleValGlyProArgThrCysArgAsnMetTrpSerGlyThrPhe
 AACGGACGATGAGGATCGTCGGTCTCTAGGACCTGCAGGAACATGTGGAGTGGACCTTC
 TTGCCCTGCTACTCCTAGCAGCCAGGATCCTGGACGTCTCTGTACACCTCACCCCTGGAAG

 6181 ProIleAsnAlaTyrThrThrGlyProCysThrProLeuProAlaProAsnTyrThrPhe
 CCCATTATGCTACACCAAGGGCCCTGTACCCCTTCTGCGCCGAACTACACGTTC
 GGGTAATTACGGATGTGGTCCCGGGACATGGGGGGAAGGACCGCGCTTGATGTGCAAG

 6241 AlaLeuTrpArgValSerAlaGluGluTyrValGluIleArgGlnValGlyAspPheHis
 GCGCTATGGAGGTGTCTGCAGAGGAATATGTGGAGATAGGACAGGTGGGGACTTCCAC
 CGCGATACCTCCACAGACGTCTCCTTATACACCTCTATTCCGTCCACCCCTGAAGGTG

 6301 TyrValThrGlyMetThrThrAspAsnLeuLysCysProCysGlnValProSerProGlu
 TACGTGACGGGTATGACTACTGACAACTCAAAATGCCCGTGCAGGTCCCATCGCCCGAA
 ATGCACTGCCCCATACTGATGACTGTAGAGTTTACGGGCAACGGTCCAGGGTAGCGGGCTT

18/ 26

6361 PhePheThrGluLeuAspGlyValArgLeuHisArgPheAlaProProCysLysProLeu
TTTTTCACAGAAATTGGACGGGTGCGCTACATAGGTTTGCGCCCCCTGCAAGCCCTTG
AAAAAGTGTCTTAACCTGCCCCACGCGGATGTATCCAAACGCGGGGGACGTTCCGGGAAC

6421 LeuArgGluGluValSerPheArgValGlyLeuHisGluTyrProValGlySerGlnLeu
CTGCGGGAGGAGGTATCATTCAGAGTAGGACTCCACGAATACCCGGTAGGGTCGCAATTA
GACGCCCTCCTCCATAGTAAGTCTCATCTCAGGTGCTTATGGGCCATCCCAGCGTTAAT

6481 ProCysGluProGluProAspValAlaValLeuThrSerMetLeuThrAspProSerHis
CCTTGGAGCCCCGAACCGGACGTGGCCGTGTTGACGTCCATGCTCACTGATCCCTCCCAT
GGAACGCTCGGGCTTGGCCTGCACCGGCACAACTGCAGGTACGAGTACTAGGGAGGGTA

6541 IleThrAlaGluAlaAlaGlyArgArgLeuAlaArgGlySerProProSerValAlaSer
ATAACAGCAGAGCGCGCGCGAAGGTTGGCGAGGGATCACCCCTCTGTGGCCAGC
TATTGTCGTCTCCGCGCGCCGCTTCCAAACCGCTCCCTAGTGGGGGAGACACCGGTCTG

6601 SerSerAlaSerGlnLeuSerAlaProSerLeuLysAlaThrCysThrAlaAsnHisAsp
TCCTCGGCTAGCCAGCTATCCGCTCCATCTCTCAAGGCAACTTGCAACCGCTAACCATGAC
AGGAGCCGATCGGTCCGATAGCGGAGGTAGAGAGTCCGTTGAACGTGGCGATTGGTACTG

6661 SerProAspAlaGluLeuIleGluAlaAsnLeuLeuTrpArgGlnGluMetGlyGlyAsn
TCCCTGATGCTGAGCTCATAGAGGCCAACCTCCTATGGAGGCAGGAGATGGGCGGCAAC
AGGGGACTACGACTCGAGTATCTCCGGTTGGAGGATACCTCCGTCTCTACCCCGCGTTG

FIG. 1R

19 / 26

FIG. 1S

IleThrArgValGluSerGluAsnLysValValIleLeuAspSerPheAspProLeuVal
 6721 ATCACCCAGGGTTGAGTCAGAAAAACAAAGTGGTATTCTGGACTCCTTCGATCCGCTTG
 TAGTGGTCCCAACTCAGTCTTTGTTTCAACCACTAAGACCTGAGGAAGCTAGGCGAACAC

 AlaGluAspGluArgGluIleSerValProAlaGluIleLeuArgLysSerArgArg
 6781 GCGGAGGAGGACGAGCGGAGATCTCCGTACCCGCAGAAATCCTGCGGAAGTCTCGGAGA
 CGCCTCCTCCTGCTCGCCCTCTAGAGGCATGGCGCTCTTTAGGACGCCCTTCAGAGCCTCT

 PheAlaGlnAlaLeuProValTrpAlaArgProAspTyrAsnProProLeuValGluThr
 6841 TTCCGCCAGGCCCTGCCCTTTGGCGCGCGGCGGACTATAACCCCGCTAGTGGAGACG
 AAGCGGTCCGGACGGGCAACCCGCGCGCTGATATTGGGGCGGATCACCTCTGC

 TrpLysLysProAspTyrGluProProValValHisGlyCysProLeuProProLys
 6901 TGGAAAAAGCCCGACTACGAACCACTGTGTCCATGGCTGTCCGCTTCCACCTCCAAAG
 ACCTTTTTCGGGCTGATGCTTGGTGACACCAAGGTACCGACAGGCGAAGGTGGAGTTTC

 SerProProValProProArgLysLysArgThrValValLeuThrGluSerThrLeu
 6961 TCCCTCCTGTGCTCCGCTCGGAAGAACGCGACGGTGGTCCCTCACTGAATCAACCCTA
 AGGGAGGACACGGAGCGGAGCCTTCTTCCCTGCCACCCAGGAGTACTAGTTGGGAT

 (Ser)
 SerThrAlaLeuAlaGluLeuAlaThrArgSerPheGlySerSerSerThrSerglyIle
 7021 TCTACTGCCCTTGGCCGAGCTCGCCACAGAAAGCTTTGGCAGCTCCTCAACTCCGGCAT
 AGATGACGGAACCGGCTCGAGCGGTGCTTCGAAACCGTCGAGGAGTTGAAGGCCGTAA

 ThrGlyAspAsnThrThrThrSerSerGluProAlaProSerGlyCysProProAspSer
 7081 ACGGCGGACAAATACGACAAACATCCTCTGAGCCCGCCCTTCTGGCTGCCCGGACTCC
 TGCCCCGCTGTATGCTGTGTAGGAGACTCGGGCGGGGAAGACCGACGGGGGGCTGAGG

20/26

(PheAla)
7141 AspAlaGluSerTyrSerSerMetProProLeuGluGlyGluProGlyAspProAspLeu
GACGCTGAGTCCTATTCTCCTCATGCCCTCCCTGGAGGGGAGCCTGGGATCCGGATCTT
CTGCGACTCAGGATAAGGAGGTACGGGGGGACCTCCCTCGGACCCCTAGGCCCTAGAA

7201 SerAspGlySerTrpSerThrValSerSerGluAlaAsnAlaGluAspValValCysCys
AGCGACGGGTCAATGGTCAACGGTCAGTAGTAGGCCCAACCGGAGGATGTCGTGTCTGC
TCGCTGCCCAGTACCAGTTGCCAGTCACTCACTCCGGTTGGCCTCCTACAGCACACGACG

7261 SerMetSerTyrSerTrpThrGlyAlaLeuValThrProCysAlaAlaGluGlnLys
TCAATGTCTTACTCTTGGACAGCGGCACTCGTCACCCCTGCGCGGAAAGAACAGAAA
AGTTACAGAATGAGAACCTGTCCCGTGAGCAGTGGGCGCACGGCGCCTTCTGTCTTT

7321 LeuProIleAsnAlaLeuSerAsnSerLeuLeuArgHisAsnLeuValTyrSerThr
CTGCCCATCAATGCACATAAGCAACTCGTTGCTACGTCAACCAATTTGGTGATTTCCACC
GACGGGTAGTTACGTGATTCTGTTGAGCAACGATGCAGTGGTGTAAACCAATAAGGTGG

7381 ThrSerArgSerAlaCysGlnArgGlnLysLysValThrPheAspArgLeuGlnValLeu
ACCTCAGCGCAGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
TGGAGTGGCTCAGCAACGGTTTCCGCTCTTCTTCTCAGTGTAAACTGTCTGACGTTCAAGAC

7441 AspSerHisTyrGlnAspValLeuLysGluValLysAlaAlaAlaSerLysValLysAla
GACAGCCATTACCAGGACGTACTCAAGGAGGTAAAGCAGCGCGTCAAAAGTGAAGGCT
CTGTCCGGTAATGGTCCCTGCATGAGTTCCTCCAATTTCTGTCGCCGCGAGTTTCACTTCCGA

FIG. 1T

21 / 26

FIG. 1U

(Phe)

7501 AsnLeuLeuSerValGluGluAlaCysSerLeuThrProProHisSerAlaLysSerLys
AACTTGCTATCCGTAGAGGAAGCTTGACGCTGACGCCCCACACTCAGCCAAATCCAAG
TTGAACGATAGGCATCTCCTTCGAACGTCGACTGCGGGGTGTGAGTCGGTTAGGTTC

7561 PheGlyTyrGlyAlaLysAspValArgCysHisAlaArgLysAlaValThrHisIleAsn
TTTGTTATGGGGCAAAAGACGTCCGTTGCCATGCCAGAAAGCCGTAACCCACATCAAC
AAACCAATACCCCGTTTCTGCAGGCAACGGTACGGTCTTTCGGGCATTGGGTGTAGTTG

7621 SerValTrpLysAspLeuLeuGluAspAsnValThrProIleAspThrThrIleMetAla
TCCGTGTGGAAGACCTTCTGGAAGACAATGTAAACACCAATAGACACTACCATCATGGCT
AGGCACACCTTCTGGAAGACCTTCTGTATCATTTGTGGTTATCTGTGATGGTAGTACCGA

7681 LysAsnGluValPheCysValGlnProGluLysGlyGlyArgLysProAlaArgLeuIle
AAGAACGAGGTTTCTGCGTTCAGCCTGAGAAGGGGGTTCGTAAGCCAGCTCGTCTCATC
TTCCTTGCTCCAAAGACGCAAGTCGGACTCTTCCCCCAGCATTCGGTCCGAGCAGAGTAG

7741 ValPheProAspLeuGlyValArgValCysGluLysMetAlaLeuTyrAspValValThr
GTGTTCCCCGATCTGGCGGTGCGCGTGTGCCGAAAGATGGCTTTGTACGACGTGGTTACA
CACAAAGGGCTAGACCCCGCACGCGCACACGCTTTTCTACCGAAACATGCTGCACCAATGT

7801 LysLeuProLeuAlaValMetGlySerSerTyrGlyPheGlnTyrSerProGlyGlnArg
AAGCTCCCCCTTGGCCGTGATGGGAAGCTCCTACGGATTCCAATACTACCCAGGACAGCGG
TTCGAGGGGAACCGGCACTACCTTCGAGGATGCCCTAAGGTTATGAGTGTCTGTCTGCC

7861 ValGluPheLeuValGlnAlaTrpLysSerLysLysThrProMetGlyPheSerTyrAsp
GTTGAATTCTCTCGTCAAGCGTGGGAAGTCCAAGAAAACCCCAATGGGGTTCTCGTATGAT
CAACTTAAGGAGACGTTTCGCACCTTCAGGTTCTTTTGGGGTTACCCCAAGAGCATACTA

23/ 26

FIG. 1W

8281 GluAlaMetThrArgTyrSerAlaProProGlyAspProProGlnProGluTyrAspLeu
GAGGCTATGACCAGGTACTCCGCCCCCTGGGACCCCCACAAACCAGAAATACGACTTG
CTCCGATACTGGTCCATGAGCGGGGGACCCCTGGGGGGTGTGGTCTTATGCTGAAC

8341 GluLeuIleThrSerCysSerSerAsnValSerValAlaHisAspGlyAlaGlyLysArg
GAGCTCATAACATCATGCTCCTCCACGTTGTAGTCGCCCCACGACGGCGCTGGAAGAGG
CTCGAGTATTGTAGTACGAGGAGGTGTCACAGTCAGCGGGTGTCTCCCGGACCTTTCTCC

8401 ValTyrTyrLeuThrArgAspProThrThrProLeuAlaArgAlaAlaTrpGluThrAla
GTCTACTACCTCACCCGTGACCCCTACAACCCCTCGCGAGAGCTGCGTGGGAGACAGCA
CAGATGATGGAGTGGGCACTGGGATGTTGGGGGAGCGCTCTCGACGCCCTCTGTCTCGT

8461 ArgHisThrProValAsnSerTrpLeuGlyAsnIleIleMetPheAlaProThrLeuTrp
AGACACACTCCAGTCAATTCTGGCTAGGCAACATAATCATGTGTTGCCCCACACTGTGG
TCTGTGTGAGGTCAGTTAAGGACCGATCCGTTGTATTAGTACAAACGGGGGTGTGACACC

8521 AlaArgMetIleLeuMetThrHisPhePheSerValLeuIleAlaArgAspGlnLeuGlu
CGGAGGATGATGATGATGACCCATTCTTTAGCGTCTTATAGCCAGGACCCAGCTTGAA
CGCTCCTACTATGACTACTGGGTAAAGAAATCGCAGGAATATCGGTCCCTGGTCGAACTT

8581 GlnAlaLeuAspCysGluIleTyrGlyAlaCysTyrSerIleGluProLeuAspLeuPro
CAGGCCCTCGATTGCGAGATCTACGGGGCTGCTACTCCATAGAACCACTTGATCTACCT
GTCCGGGAGCTAACGCTCTAGATGCCCCGGACGATGAGGTATCTTGGTGAAC TAGATGGA

8641 ProIleIleGlnArgLeuHisGlyLeuSerAlaPheSerLeuHisSerTyrSerProGly
CCAATCATTCAAAGACTCCATGGCCCTCAGCGCATTTTCACTCCACAGTTACTCTCCAGGT
GGTTAGTAAGTTTCTGAGGTACCGGAGTCGGGTAAAGTGAGGTGTCAATGAGAGGTCCA

24/ 26

8701 GluIleAsnArgValAlaAlaCysLeuArgLysLeuGlyValProProLeuArgAlaTrp
GAAATTAATAGGTGGCCGATGCTCAGAAACTTGGGGTACCGCCCTTGCAGACTTG
CTTTAATTATCCACCGCGGTACGGAGTCTTTTGAACCCCATGGCGGAACGCTCGAACC

Gly
8761 ArgHisArgAlaArgSerValArgAlaArgLeuLeuAlaArgGlyGlyArgAlaIle
AGACACCGGCGCGAGCGTCCGCGCTAGGCTTCTGGCCAGAGGAGCGGCTGCCATA
TCTGTGGCCCGGCGCTCGCAGGCGCATCCGAAGACCGGTCTCTCCGTCCCGACGGTAT

8821 CysGlyLysTyrLeuPheAsnTrpAlaValArgThrLysLeuLysLeuThrProIleAla
TGTGGCAAGTACCTCTTCAACTGGCGAGTAAGAACAAGCTCAAACTCACTCCAATAGCG
ACACCGTTTCATGGAGAACTTGACCCGTCATTCTTGTTCGAGTTTGAGTGAGGTTATCGC

8881 AlaAlaGlyGlnLeuAspLeuSerGlyTrpPheThrAlaGlyTyrSerGlyGlyAspIle
GCCGCTGGCCAGCTGGACTTGTCCGGCTGGTTCACGGCTGGCTACAGCGGGGAGACATT
CGCGACCGGTCCGACCTGAACAGGCCGACCAAGTGCCGACCGATGTGCCCCCTCTGTAA

(Pro)
8941 TyrHisSerValSerHisAlaArgProArgTrpIleTrpPheCysLeuLeuLeuAla
TATCACAGCGTGTCTCATGCCCGGCCCGCTGGATCTGGTTTTGCTACTCCTGCTGCT
ATAGTGTGCACAGAGTACGGCGCGGCGACCTAGACCAAAACGGATGAGGACGAACGA

FIG. 1X

25/ 26

FIG. 1Y

AlaGlyValGlyIleTyrLeuLeuProAsnArgOP
GCAGGGGTAGGCATCTACCTCCTCCCAACCGATGAAGGTGGGGTAAACACTCCGGCCT
CGTCCCATCCGTAATGAGGAGGGGTGGCTACTTCCAAACCCCATTTGTGAGGCCCGGA

9001

() = Heterogeneity due possibly to 5' or 3' -
terminal cloning artefact

26/26

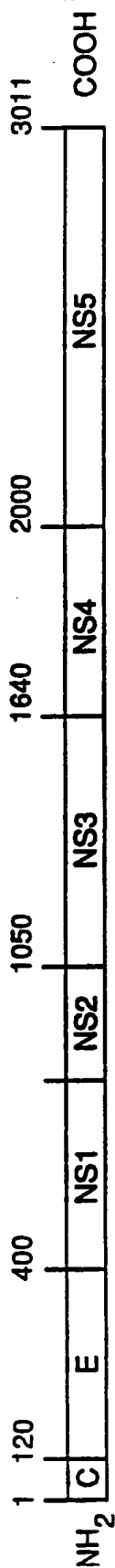


FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/02225

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : G 01 N 33/576, C 07 K 15/00														
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 5px;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 25%; border: none;">Classification System</td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">IPC⁵</td> <td style="border: 1px solid black; padding: 5px;">G 01 N, C 07 K</td> </tr> </table> <div style="text-align: center; margin-top: 5px; font-size: small;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁵	G 01 N, C 07 K								
Classification System	Classification Symbols													
IPC ⁵	G 01 N, C 07 K													
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; text-align: left; padding: 5px;">Category ⁹</th> <th style="width: 70%; text-align: left; padding: 5px;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; text-align: left; padding: 5px;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;"> WO, A, 8904669 (CHIRON CORP.) 1 June 1989 see page 39, lines 8-12; page 49, line 5 - page 50, line 31; page 123, line 29 - page 125, line 22; page 132, line 3 - page 134, line 35; page 171, lines 4-20 <div style="text-align: center;">--</div> </td> <td style="vertical-align: top; padding: 5px;">1-16</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;"> EP, A, 0318216 (CHIRON CORP.) 31 May 1989 see page 15, line 39 - page 17, line 8; page 18, line 44 - page 19, line 13; page 27, lines 10-22 cited in the application <div style="text-align: center;">--</div> </td> <td style="vertical-align: top; padding: 5px;">1-16</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;"> Science, vol. 244, 21 April 1989, (Washington, DC, US), G. Kuo et al.: "An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis", pages 362-364 see the whole article <div style="text-align: center;">-----</div> </td> <td style="vertical-align: top; padding: 5px;">1-16</td> </tr> </tbody> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	WO, A, 8904669 (CHIRON CORP.) 1 June 1989 see page 39, lines 8-12; page 49, line 5 - page 50, line 31; page 123, line 29 - page 125, line 22; page 132, line 3 - page 134, line 35; page 171, lines 4-20 <div style="text-align: center;">--</div>	1-16	Y	EP, A, 0318216 (CHIRON CORP.) 31 May 1989 see page 15, line 39 - page 17, line 8; page 18, line 44 - page 19, line 13; page 27, lines 10-22 cited in the application <div style="text-align: center;">--</div>	1-16	A	Science, vol. 244, 21 April 1989, (Washington, DC, US), G. Kuo et al.: "An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis", pages 362-364 see the whole article <div style="text-align: center;">-----</div>	1-16
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
Y	WO, A, 8904669 (CHIRON CORP.) 1 June 1989 see page 39, lines 8-12; page 49, line 5 - page 50, line 31; page 123, line 29 - page 125, line 22; page 132, line 3 - page 134, line 35; page 171, lines 4-20 <div style="text-align: center;">--</div>	1-16												
Y	EP, A, 0318216 (CHIRON CORP.) 31 May 1989 see page 15, line 39 - page 17, line 8; page 18, line 44 - page 19, line 13; page 27, lines 10-22 cited in the application <div style="text-align: center;">--</div>	1-16												
A	Science, vol. 244, 21 April 1989, (Washington, DC, US), G. Kuo et al.: "An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis", pages 362-364 see the whole article <div style="text-align: center;">-----</div>	1-16												
<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;"> Date of the Actual Completion of the International Search <div style="border: 1px solid black; padding: 2px; display: inline-block;">5th July 1991</div> </td> <td style="width: 50%; border: none;"> Date of Mailing of this International Search Report <div style="border: 1px solid black; padding: 2px; display: inline-block;">- 2. 09. 91</div> </td> </tr> <tr> <td style="border: none;"> International Searching Authority <div style="border: 1px solid black; padding: 2px; display: inline-block;">EUROPEAN PATENT OFFICE</div> </td> <td style="border: none;"> Signature of Authorized Officer <div style="border: 1px solid black; padding: 2px; display: inline-block;">M. PEIS</div> <div style="margin-left: 20px;"> </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="border: 1px solid black; padding: 2px; display: inline-block;">5th July 1991</div>	Date of Mailing of this International Search Report <div style="border: 1px solid black; padding: 2px; display: inline-block;">- 2. 09. 91</div>	International Searching Authority <div style="border: 1px solid black; padding: 2px; display: inline-block;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="border: 1px solid black; padding: 2px; display: inline-block;">M. PEIS</div> <div style="margin-left: 20px;"> </div>								
Date of the Actual Completion of the International Search <div style="border: 1px solid black; padding: 2px; display: inline-block;">5th July 1991</div>	Date of Mailing of this International Search Report <div style="border: 1px solid black; padding: 2px; display: inline-block;">- 2. 09. 91</div>													
International Searching Authority <div style="border: 1px solid black; padding: 2px; display: inline-block;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="border: 1px solid black; padding: 2px; display: inline-block;">M. PEIS</div> <div style="margin-left: 20px;"> </div>													

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9102225
SA 46573

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 27/08/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8904669	01-06-89	AU-A- 2796789	14-06-89
		EP-A- 0318216	31-05-89
		GB-A- 2212511	26-07-89
		JP-T- 2500880	29-03-90

EP-A- 0318216	31-05-89	AU-A- 2796789	14-06-89
		GB-A- 2212511	26-07-89
		JP-T- 2500880	29-03-90
		WO-A- 8904669	01-06-89

EPO FORM PWT9

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82